Interaction between SQSTM1/p62 and HDAC6: Impacts on the Aggresome-autophagy Pathway and Microtubule Dynamics

by

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Abstract

Aggresome-autophagy is a cellular degradation pathway that responds to protein misfolding stress. This pathway is particularly important because it maintains cellular homeostasis whenever the ubiquitin-proteasome degradation pathway is impaired. Misfolded proteins processed by the aggresome-autophagy pathway are first accumulated at the microtubule organizing center (MTOC) through retrograde transport. Proteins are formed into aggresomes which are then targeted by autophagic machinery for lysosome-dependent degradation. Research in recent years has revealed that the class II histone deacetylase HDAC6 plays important roles in regulation of aggresome formation and autophagic degradation. This regulation is achieved by interplay between HDAC6 and its interacting proteins. Interestingly, our laboratory's long-time interest SQSTM1/p62 has also been shown to be critical for aggregation of misfolded proteins and their autophagic degradation. Similar to HDAC6, p62 also has a C-terminal ubiquitin-binding domain. It has been documented that HDAC6 and p62 may work together in regulation of aggresome-autophagy pathway. In Chapter 1 of this dissertation, I review the molecular mechanisms involved in this regulation and the literature related to HDAC6 and p62. In Chapter II, I further review the structure and function of HDAC6.

Based on the current literature regarding the roles of HDAC6 and p62 in the aggresome-autophagy pathway, I hypothesized that p62 may directly interact with HDAC6. I further hypothesized that any such interaction might alter HDAC6 activity as well as impact its

regulation of the aggresome-autophagy pathway and, potentially, microtubule dynamics. In order to investigate these hypotheses, two objectives are undertaken: (1) I conducted research aimed at identifing the possible interaction between p62 and HDAC6. Under this objective, I examined the functional effect on HDAC6 activity and the aggresome-autophagy pathway caused by loss of this interaction in p62KO mouse embryonic fibroblasts (MEFs); (2) I examined a hypothesized association between p62 and microtubules. Included in this objective was research aimed at identifying potential microtubule dynamic properties caused by altered HDAC6 activity observed in p62KO MEFs. In Chapter III, I show, using both biochemical and immunofluorescence approaches, that p62 indeed interact with HDAC6. Using deletion mutants, I mapped the interaction site on p62 to a region between the ZZ domain and TRAF6 domains. Using a similar approach, I found that the DD2 region of HDAC6 was the most likely site for its interaction with p62. I discovered that lack of this interaction in p62KO MEFs led to ***increased*** HDAC6 activity and ***reduced acetylation*** of two important HDAC6 substrates, α-tubulin and cortactin. I further show that p62 is essential for assembly of cortactin-actin networks at aggresome-like aggregates containing HDAC6 which subsequently plays a critical role during autophagy degradation of aggresomes. Overall, these results indicate that a loss of this interaction may impair the aggresome-autophagy pathway. In Chapter IV, I show, using both biochemical and immunofluoresence approaches, that p62 associates with microtubules. Absence of p62 in MEFs results in microtubule stabilization. However, pharmacological inhibition of HDAC6 in these cells did not stabilize microtubules, but enhanced binding of HDAC6 to microtubules. I also show that lack of p62 in MEFs leads to increased rate of microtubule reassembly compared to wild-type conditions. Collectively, these results indicate that microtubules from p62KO MEFs have different dynamic properties compared to those in wild-

type cells. I conclude that both p62 deficiency and elevation of HDAC6 activity may contribute to altered microtubule dynamics in p62KO MEFs.

Future studies towards better understanding "how" p62 interacts with HDAC6 were discussed in Chapter V. First, it would be meaningful to identify the residues on HDAC6 responsible for this interaction and regulation. Second, it would be imperative to explore possible functional redundancy between p62 and HDAC6 in the aggresome-autophagy pathway since HDAC6 activity is elevated with absence of p62 in MEFs. Third, it would also be worthwhile to examine the possible model in which p62 and HDAC6 may work together to recruit protein cargoes for aggresome formation. Last, it would be interesting to investigate whether decreased acetylated tubulin in p62 null background results in impaired microtubule trafficking which can contribute to Alzheimer's Disease-like phenotype.

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CHAPTER I. LITERATURE REVIEW: ROLES OF HDAC6 AND ITS INTEERACTING PARTNERS IN AGGRESOME-AUTOPHAGY PATHWAY AND NEURODEGENERATION

Introduction

Almost 30% of newly synthesized proteins are not properly folded, thus production of misfolded proteins is common during biogenesis. Protein misfolding is particularly exacerbated in cells experiencing diseased states, such as neurodegeneration, due to conformational change caused by protein mutation (Ross and Poirier, 2004). Consequently, misfolded proteins often accumulate and form toxic aggregates in such cells. In response, cells have evolved defense mechanisms to remove aggregates and prevent toxicity. The ubiquitin-proteasome system (UPS) is the conventional pathway by which misfolded protein aggregates are disposed of (Lilienbaum, 2013). This pathway relies on tagging of misfolded proteins with ubiquitin and proteolytic degradation by the proteasome. However, in several neurodegenerative diseases, UPS becomes dysfunctional (Paul, 2008). Therefore, an alternative degradation pathway needs to step up to compensate UPS defect. One of these pathways is aggresome-autophagy. In this pathway, misfolded protein aggregates move along microtubules to the microtubule organizing center (MTOC) to form a large aggregate (aggresome) (Olzmann et al., 2008).

Aggresomes attenuate toxicity of dispersed cytoplasmic aggregates by accumulating them at one place within the cell (Yao, 2010). In the next step of this pathway, lysosome-dependent autophagy machinery is recruited to aggresomes for aggregate clearance. Studies in recent years revealed that the aggresome-autophagy pathway plays a critical role in selective removal of ubiquitinated protein aggregates (Iwata et al., 2005b, Olzmann et al., 2007).Research in the last decade has also shown that histone deacetylase 6 (HDAC6) is a crucial component of the aggresome-autophagy pathway (Yao, 2010). As a unique member of the histone deacetylase family, HDAC6 is generally localized in the cytoplasm of the cell allowing it to interact with a number of cytoplasmic proteins (Verdel et al., 2000). Several of these HDAC6 interacting proteins have also been shown to be involved in the aggresome-autophagy pathway. This review paper will focus on outlining the molecular and signaling mechanisms by which HDAC6 and its interacting proteins regulate each individual step in this degradation pathway. I also seek to provide perspectives on potential target in this pathway that may prove important for treatment of neurodegenerative diseases.

UPS: Degradation of Ubiquitinated Misfolded Proteins

Ubiquitin is a small protein of 76 amino acids. It can be covalently attached to the target protein by forming a isopeptide bond between C-terminal glycine of ubiquitin and ε-amino group of the lysine residue on target protein. This process is named ubiquitination which is one type of common post-translational modification. Ubiquitination is catalyzed by a cascade of three ubiquitin enzymes, namely E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-ligases

(Kerscher et al., 2006). Proteins can be modulated by a single ubiquitin (monoubiquitination) or a linear chain of ubiquitins (polyubiquitination). There are 7 different types of lysine linkage (K6, K11, K27, K29, K33, K48, and K63) in identified polyubiquitin chains (Peng et al., 2003). Of these linkages, K48-linked polyubiquitin chains are specifically associated with targeting proteins for proteasomal degradation (Chau et al., 1989, Thrower et al., 2000).

Ubiquitination plays an important role in targeting cellular proteins for proteasomal degradation (Tanahashi et al., 1999, Voges et al., 1999). Misfolded proteins are recognized by a cellular quality control system. This system recruits E3 ubiquitin ligase to tag these proteins with a ubiquitin moiety leading to recognition by specific shuttling factors with ubiquitin-binding domain that promote shuttling to 26S proteasomes. In a final step, ubiquitinated proteins are deubiquitinated by deubiquitinases (DUBs) and unfolded by unfoldase before entering the proteolytic lumen of 26S proteasome (Tai and Schuman, 2008). Cells have fine tuned control of each component in the UPS in order to promote efficient degradation of misfolded proteins. In the diseased state, such as neurodegeneration, one or more components of the UPS are frequently impaired (Dennissen et al., 2012). As a result, UPS-mediated degradation is compromised leading to accumulation of misfolded proteins (Fortun et al., 2005). Accumulation of misfolded proteins in cells in turn negatively affect UPS function, such as proteasome activity, thus resulting in further accumulation (Kristiansen et al., 2007).

Aggresomes: Accumulation of Protein Aggregates at MTOC

When misfolded proteins somehow escape or exceed the capacity of quality-control systems, they tend to expose hydrophobic surfaces which are usually embedded in the interior of the protein or at the interface of other subunits (Wetzel, 1994). This inappropriate exposure can result in changes to protein conformations that in turn facilitate the interaction between monomers to form aggregates. Aggregates are generally considered to be insoluble, thus are difficult to degrade (Johnston et al., 1998). Frequently, protein aggregates negatively affect proteasome activity by an undefined mechanism which leads to further aggregation of misfolded proteins. Therefore, protein aggregates are commonly cytotoxic (Ross and Poirier, 2005).

Fortunately, cells respond to aggregates by stimulating an alternative degradation pathway. Upon accumulation, aggregates are moved along microtubules to the microtubule organizing center (MTOC) to form a juxtanuclear structure named "aggresome" (Johnston et al., 1998). Johnston et al. first discovered aggresome formation of two misfolded membrane proteins, cystic fibrosis conductance regulator (CFTR) and presenilin-1 (PS1) (Johnston et al., 1998). Either overexpression of CFTR or PS1 or pharmacological inhibition of proteasome induces aggresome formation. Ubiquitin was detected as an aggresome protein as misfolded proteins are normally tagged by ubiquitin (Johnston et al., 1998). The intermediate filament (IF) protein vimentin was also visualized as a "cage-like strucuture" surrounding aggresomes. IF is believed to collapse and entangle with the aggresome at the juxtanuclear region of the cell (Johnston et al., 1998). It was proposed that formation of aggresomes are cytoprotective as they remove

toxic protein aggregates from cytoplasm and concentrate them at the MTOC (Kopito, 2000).

Fate of Aggresomes: Degradation by Autophagy

How aggregates in aggresomes are degraded has been an intense research interest since the discovery of these structures. Interestingly, proteasomes were observed to colocalize with aggresomes at the MTOC (Wigley et al., 1999, Fabunmi et al., 2000). This potentiates the possibility for degradation of aggresomes by proteasomes. However, aggregates are normally difficult to unfold, thus hard to be degraded by proteasomes.

Emerging evidence has shown that aggresomes tend to be removed by autophagy (Fortun et al., 2003, Taylor et al., 2003, Iwata et al., 2005a, Iwata et al., 2005b, Su et al., 2011). Indeed, components of the autophagy machinery have been observed to colocalize at aggresomes (Iwata et al., 2005b). Further supporting this hypothesis, clearance of aggresomes is closely associated with induction and inhibition of autophagy (Ravikumar et al., 2004, Yamamoto et al., 2006). The major steps of autophagy include autophagosome formation and autophagosome-lysosome fusion (Mizushima, 2007). Autophagic substrates are engulfed when autophagosomes are formed and are finally degraded by autolysosomes formed from autophagosome-lysosome fusion (Mizushima, 2007). Originally identified as bulk-digestion process, the classical autophagy removes cytoplasmic proteins and organelles in a non-selective manner (Cuervo, 2008). This type of autophagy is often induced by nutrient-starvation to replenish amino acid deficiency (Cuervo, 2008). In recent years, selective autophagy has gained research attention(Kraft et al., 2010). This type of autophagy often selectively targets ubiquitin-tagged protein,

and is thus able to distinguish toxic protein aggregates from normal cellular counterparts (Kraft et al., 2010). This type of autophagy is believed to degrade ubiquitinated aggregates collected at aggresomes and is therefore considered a form of quality-control autophagy (Yao, 2010).

Domain Structure of HDAC6 and Diversity of Its interacting Partners

HDAC6 is a member of the class II histone deacetylase family. An N-terminal NES translocates HDAC6 from the nucleus to the cytoplasm (Verdel et al., 2000). Several functional domains have been identified in HDAC6. Two critical domain, DD1 and DD2 are involved in HDAC6's deacetylase activity (Hubbert et al., 2002, Zhang et al., 2006). A dynein binding domain localized between DD1 and DD2 specifically interacts with motor protein dynein (Kawaguchi et al., 2003). In addition, a C-terminal BUZ/ZnF-UBP domain binds both mono- and poly- ubiquitin, thus connecting HDAC6 with ubiquitin-dependent signaling pathways (Seigneurin-Berny et al., 2001, Boyault et al., 2006). In fact, the BUZ domain has been shown to bind mono ubiquitin with the highest affinity among the ubiquitin-binding domains so far identified (Boyault et al., 2006). Human HDAC6 also contains a unique SE14 domain localized between the DD2 and BUZ domain that serves to anchor the protein in the cytoplasm (Bertos et al., 2004).

HDAC6-interacting partners refer to proteins that physically interact with HDAC6 or indirectly associate with HDAC6 in a protein complex. These can be HDAC6 cytoplasmic substrates, such as α-tubulin, cortactin and Hsp90 (Zhang et al., 2003, Kovacs et al., 2005, Zhang et al., 2007). Acetylation of these substrates controlled by HDAC6 contribute to cytoskeleton-dependent and chaperone-dependent cellular

functions (Kovacs et al., 2005, Zhang et al., 2007, Zilberman et al., 2009). HDAC6 interacting partners also regulate HDAC6 function through modifying HDAC6 catalytic activity. This regulation can be achieved by physical interaction or post-translational modification on HDAC6. The third way HDAC6-interacting partners behave is to form a signaling complex with HDAC6 to work synergistically. This type of complex generally does not regulate HDAC6 activity, but instead links HDAC6 to other signaling proteins. Therefore, the interplay between HDAC6 and its interacting partners plays an important role in HDAC6-dependent cellular pathways.

HDAC6 and Dynein: the Central Component of Aggresome Assembly Machinery

Intact microtubules are indispensible for aggresome formation (Johnston et al., 1998). In support of this role, aggresome formation is abrogated in cells treated with microtubule depolymerizer nocodazole (Johnston et al., 1998). Johnston et al. further studied the mechanism by which aggregates are moved along microtubules to form aggresomes. They showed that a dynein/dynactin complex is responsible for moving aggregate cargoes to the MTOC (Johnston et al., 2002). A subsequent investigation by Kawaguchi et al. (Kawaguchi et al., 2003) revealed that HDAC6 is essential for dynein to move polyubiquitinated protein aggregates to form aggresomes. Through interaction with both polyubiquitinated aggregates and dynein, HDAC6 serves as an adaptor between these two to form cargo-HDAC6-dynein complexes. In support of HDAC6's role in bridging aggregates and dynein for aggresome formation, knockdown of HDAC6 in cells has been shown to lead to aggresome-deficiency (Kawaguchi et al., 2003). Interestingly, catalytic inactive HDAC6 mutants fail to restore aggresome formation in HDAC6

knockdown cells indicating that HDAC6 activity is also involved in this process. This result suggests that the HDAC6-dynein complex may be a central component for directing movement of polyubiquitinated aggregates to the MTOC.

HDAC6 and Parkin: Ubiquitination Signal for Aggresome formation

Kawaguchi et al. (Kawaguchi et al., 2003) examined the HDAC6-dynein complex in an ubiquitinated CFTRFΔ508 aggresome model. This raises the question of which specific E3 ubiquitin ligase is involved in the generation of ubiquitination signals for recognition of cargo. A breakthrough was made by studying mutant DJ-1, a protein ubiquitously expressed in early-onset Parkinson's disease (Olzmann et al., 2007). They showed that transport of mutant DJ-1 to form aggresomes is also directed by the HDAC6-dynein complex. The E3 ligase parkin was shown to mediate Lys-63 ubiquitination of mutant DJ-1 for recognition by HDAC6. In parkin-deficient cells, lack of Lys-63 ubiquitination prevents assembly of the cargo-HDAC6-dynein complex which leads to failure of aggresome formation (Olzmann et al., 2007).

Intriguingly, parkin itself can accumulate at centrosome regions upon proteasome inhibition (Zhao et al., 2003). This activity seems to resemble accumulation of protein aggregates at aggresomes and implies that centrosomal recruitment of parkin also relies on the HDAC6-dynein complex. Through direct interaction parkin, HDAC6, and dynein form a complex that moves to the minus end of microtubules to accumulate at the centrosome (Jiang et al., 2008). The catalytic activity of HDAC6 is critical for this recruitment as evidenced by abolished centrosomal accumulation of parkin upon HDAC6 inhibition by tubacin. As parkin remains catalytically active along its way to the

centrosome, it is possible that the presence of a parkin-HDAC6 complex could facilitate further ubiquitination of protein aggregates bound by HDAC6. This activity may act to stabilize the cargo-HDAC6-dynein complex during its movement to the MTOC (Jiang et al., 2008). In addition, parkin was found to ubiquitinate mutant TDP-43, a nuclear protein that can accumulate to form cytoplasmic inclusions in several neurodegenerative diseases. This ubiquitination signal triggers binding of TDP-43 to the HDAC6 complex leading to its cytoplasmic inclusion formation (Hebron et al., 2013).

HDAC6 and Ataxin: Generation of Unanchored Ubiquitin for Aggresome Formation

The efficient recruitment of aggregates is also dependent on the binding efficiency of HDAC6's BUZ domain in relation to polyubiquitin chains on protein aggregates. Ubiquitination is controlled by the balance of ubiquitinating enzymes and deubiquitinases (DUBs)(Finley, 2009). Interestingly, analogs of the BUZ domain of HDAC6 are often found in deubiquitinases that bind unanchored ubiquitin C-termini (Reyes-Turcu et al., 2006, Pai et al., 2007). Interestingly, unanchored ubiquitin C-termini are not present in polyubiquitin chains as ubiquitin moieties are conjugated through a C-terminal diglycine motif. Thus, it is impossible for HDAC6 to bind polyubiquitin chains without the presence of unanchored C-termini.

Advances in understanding this puzzle were first achieved by identification the role of DUB in aggresome formation. Deubiquitinase ataxin-3 was found to colocalize with aggresomes formed by CFTRΔF508 and associate with HDAC6 and dynein (Burnett and Pittman, 2005). Knockdown of ataxin-3 in cells significantly decreases CFTRΔF508

aggresome formation indicating that ataxin-3 is essential for this process (Burnett and Pittman, 2005). One important function of DUB is editing of polyubiquitin chains. A detailed model of how ataxin-3 regulates aggresome formation was elucidated in a recent study. Ataxin-3 is required for generation of unanchored ubiquitin C-termini present in polyubiquitinated CFTRΔF508 aggregates which are critical for HDAC6 binding and aggresome formation (Ouyang et al., 2012). In recruitment of CFTRΔF508 aggregates for aggresome formation, the BUZ domain of HDAC6 only binds these unanchored ubiquitin C-termini to the exclusion of conjugated polyubiquitin chains (Ouyang et al., 2012). In ataxin-3 knockdown cells, the number of unanchored ubiquitin C-termini present in CFTRΔF508 aggregates are decreased leading to compromised aggresome formation (Ouyang et al., 2012). Further supporting this role, ataxin-3 was found to regulate aggresome formation of superoxide dismutase-1 by editing Lys-63 polyubiquitin chains (Wang et al., 2012).

HDAC6 and Tau: Aggresomes Formed by Non-ubiquitinated Proteins

Interestingly, ubiqutination is not a required signal for collection of proteins at aggresome as non-ubiquitinated proteins have also been found to form aggresomes (Garcia-Mata et al., 1999, Johnston et al., 2000, Watanabe and Tanaka, 2011). Interestingly, HDAC6 is also involved in aggresome formation of the non-ubiquitinated protein tau (Guthrie and Kraemer, 2011). Tau is a microtubule-associated protein and is aggregated to form neuroflibrillary tangles in neurodegenerative diseases, such as Alzheimer's disease (Cleveland et al., 1977, Montejo de Garcini et al., 1986). HEK cells with overexpression of tau can form aggresome-like perinuclear aggregates upon

proteasome inhibition (Guthrie and Kraemer, 2011). The interaction between tau and HDAC6 was observed in both mammalian cells and mouse brain (Ding et al., 2008, Perez et al., 2009). This direct interaction connects tau with dynein, thus may enables movement of tau to the MTOC. Indeed, endogenously expressed tau colocalizes with exogenously expressed HDAC6 in aggresome-like structures found in HEK cells upon proteasome inhibition (Ding et al., 2008). Conversely, knockdown of HDAC6 in cells impairs this connection leading to abrogation of tau-associated aggresome formation(Guthrie and Kraemer, 2011).

Intriguingly, the interaction between tau and HDAC6 also inhibits HDAC6'scatalytic activity (Perez et al., 2009). Indeed, overexpression of tau in HEK cells results in hyperacetylation of tubulin, suggesting that HDAC6 activity is decreased(Perez et al., 2009). As catalytic activity of HDAC6 is also critical for maintaining function of aggresome pathways, tau overexpression also inhibits autophagy induction and autophagosome recruitment, suggesting an inhibitory effect on this pathway (Perez et al., 2009). This finding seems to be contradictory to the indispensability of HDAC6 in tau aggresome formation discussed above. However, this discrepancy may be due to different tau expression levels within the cell models used in these studies. It seems that tau-associated aggresome formation is favored by HDAC6/tau interaction in HEK cells endogenously expressing tau. The inhibitory effect on HDAC6 activity and aggresome pathway is exaggerated in HEK cells overexpressing tau. Therefore, it can be postulated that HDAC6-tau interaction may function differently in the aggresome-autophagy pathway depending on tau expression levels in cells.

HDAC6 and CK2: Phosphorylation-dependent Aggresome Formation

HDAC6 catalytic activity has been shown to be required for aggresome formation of ubiquitinated proteins, such as CFTRΔF508 and mutant DJ-1, and also non-ubiquitinated protein STAT5A_ΔE18 (Kawaguchi et al., 2003, Olzmann et al., 2007, Watanabe and Tanaka, 2011). Therefore, it is possible that HDAC6's interacting partners that regulate HDAC6 activity may affect aggresome formation. Phosphorylation has emerged as a common mechanism for HDAC6's partners to regulate HDAC6 catalytic activity. Several kinases including GSK3β, CK2, GRK2, and EGFR are reported to phosphorylate HDAC6 leading to altered catalytic activity (Deribe et al., 2009, Chen et al., 2010, Watabe and Nakaki, 2011, Lafarga et al., 2012). Of these kinases, CK2 was found to bind both dynein and microtubules (Karki et al., 1997, Faust et al., 1999, Lim et al., 2004). Through these interactions, CK2 regulates dynein phosphorylation and microtubule assembly (Karki et al., 1997, Faust et al., 1999, Lim et al., 2004). These findings suggest that CK2 may play a role in the aggresome pathway. In further support of this hypothesis, CK2 was found to directly interact with HDAC6 (Watabe and Nakaki, 2011). The CK2 phosphorylation site was localized at Ser458 which is within HDAC6's dynein binding domain (Watabe and Nakaki, 2011). Presence of CK2 is required for binding of HDAC6 to dynein *in vitro*, indicating a potential role of CK2 in modulating HDAC6-dynein complex during aggresome formation (Watabe and Nakaki, 2011).

CK2 could also impact aggresome formation by phosphorylation-dependent mediation of HDAC6 activity. A site mutagenesis study revealed that Ser458 phosphorylation is critical for maintaining activity of HDAC6. Substitution of Ser458 with Ala on HDAC6 abolished CK2 phosphorylation leading to decreased catalytic

activity and increased tubulin acetylation (Watabe and Nakaki, 2011). Conversely, replacement of Ser458 with phospho-mimic residue Glu enhanced CK2 phosphorylation resulting in increased activity and decreased tubulin acetylation (Watabe and Nakaki, 2011). CK2-mediated Ser458 phosphorylation is also crucial for HDAC6-dependent aggresome formation. While aggresomes are induced by oxidative stress in cells expressing either WT or Ser458Glu mutant HDAC6, aggresome formation is impaired by expression of Ser458Ala mutant HDAC6 (Watabe and Nakaki, 2011). Therefore, CK2 appears to function upstream to control aggresome assembly by modulating HDAC6 activity.

HDAC6, Dynein, and Microtubules: Trafficking of Autophagosome and Lysosome to Aggresomes

Aggresomes are intermediate products in the aggresome-autophagy pathway serving as "garbage bins" to accumulate protein aggregates. These accumulated protein aggregates are targeted for autophagic clearance. To enable the degradation components of autophagy machinery, such as autophagosomes and lysosomes are recruited to the MTOC following aggresome formation (Iwata et al., 2005a, Yamamoto et al., 2006). Similar to aggresome formation, recruitment of autophagsomes also relies on intact microtubule "railway" tracks, as well as, retrograde transport mediated by several crucial proteins. In support of this hypothesis, delivery of autophagosomes to aggresomes is abrogated in cells with microtubules depolymerized by nocodazole (Iwata et al., 2005b). Dynein is also a critical part of this recruitment machinery. Genetic mutations of dynein in both mouse and flies have been shown to impair autophagy activity (Ravikumar et al., 2005, Batlevi et al., 2010). Moreover, both the presence and catalytic activity of HDAC6

are involved in this recruitment process through an undefined mechanism. Either knockdown or pharmacological inhibition of HDAC6 results in failure to target autophagosomes to inclusion bodies formed by mutant Huntington (Iwata et al., 2005b). Interestingly, HDAC6 also participates in localization of lysosomes to the MTOC. In cells with knockdown of HDAC6, accumulation of lysosomes at the MTOC is compromised (Iwata et al., 2005b). Thus, HDAC6 seems to be the central part of this recruitment machinery.

HDAC6 and Cortactin: Fusion of Autophagosomes with Lysosomes.

Once autophagic components are recruited to aggresomes, aggregates are engulfed by autophagosomes as autophagic substrates. These autophagosomes are then fused with lysosomes to allow hydrolysis of protein aggregates. Interestingly, HDAC6, and its substrate cortactin, have been shown to participate in this fusion process. Lee et al. (Lee et al., 2010a) revealed that reorganization of actin filament to aggresomes is required for fusion of autophagosomes with lysosomes. This so-called "actin-remodeling" process is stimulated by deacetylation of cortactin by HDAC6. Depletion of HDAC6 in cells leads to hyperacetylation of cortactin and impaired actin remodeling (Lee et al., 2010a). As a result, autophagosome-lysosome fusion is compromised. Thus, HDAC6-driven deacetylation and cortactin-dependent actin-remodeling plays a central role in completion of autophagic clearance. This model was further expanded by identification of upstream regulator CK2 discussed above.CK2 controls cortactin-dependent actin-remodeling machinery by modulating HDAC6 activity. Expression of CK2 phospho-inactive mutant HDAC6 S458A in cells with decreased HDAC6 activity impairs

recruitment of cortactin to aggresomes(Watabe and Nakaki, 2011). These cells become less viable in responding to misfolding stress, indicating compromised autophagic clearance (Watabe and Nakaki, 2011).

HDAC6, parkin and p62: Mitochondria Aggregation and Mitophagy

In a process closely resembling aggresomal accumulation of protein aggregates, damaged mitochondria are collected at the MTOC to form large aggregates, called "mito-aggresomes"(Lee et al., 2010b). Interestingly, parkin-mediated ubiquitination signaling is also essential for mito-aggresome formation. Parkin was found to colocalize with ubiquitinated "mito-aggresomes" and was also detected in purified mitochondrial fractions(Lee et al., 2010b). In cells expressing a parkin catalytic inactive mutant, lack of ubiquitination signal resulted in abrogation of mito-aggresome formation (Lee et al., 2010b).

Recruitment of damaged mitochondria to mito-aggresomes is also directed by HDAC6-dynein mediated retrograde transport. Either HDAC6 depletion or microtubule depolymerization impairs mito-aggresome assembly (Lee et al., 2010b). Resembling autophagic clearance of protein aggregates, the completion of mitophagy is dependent upon fusion of autophagosomes with lysosomes. Both HDAC6 deacetylation and cortactin-dependent actin-remodeling are also required for this process. In cells with knockdown of either HDAC6 or cortactin, impaired actin-remodeling leads to autophagosome-lysosome fusion deficiency and abrogated mito-aggresome clearance (Lee et al., 2010b).

In studies of mito-aggresome clearance, both HDAC6 and sequestosome 1/p62 have been identified to be recruited to these ubiquitinated mitochondria (Lee et al., 2010b). While recruitment of HDAC6 is probably involved in the stimulation ofcortactin-dependent actin remodeling, the role of recruited p62 is not clear. P62 is a multi-functional signaling protein. Similar with HDAC6, p62 also has a C-terminal ubiquitin-binding domain UBA that binds polyubiquitinated proteins (Ciani et al., 2003). The function of p62 is closely associated with autophagy as evidenced by the presence of a LIR domain that directly interacts with the autophagosome marker LC3 (Pankiv et al., 2007). Through this interaction, p62 is able to target autophagosomes to its bound polyubiquitinated proteins (Pankiv et al., 2007). Thus, it is possible that p62 may alsotargetautophagosomes to mito-aggresomes for mitophagy. In support of this hypothesis, p62 knockdown in cells abrogates mito-aggresome clearance (Lee et al., 2010b).

HDAC6 and Tau in Neurodegenerative Diseases

Abnormal accumulation of tau has been found in a number of neurodegenerative diseases, particularly in Alzheimer's disease (Kosik et al., 1986). These tau proteins are usually hyperphosphorylated leading to its insolubility resulting in its aggregation (Mulvihill and Perry, 1989, Bancher et al., 1991). Aggregated tau loses its binding affinity to microtubules which destabilizes microtubules (Lu and Wood, 1993). In neurons, these tau-destabilized microtubules can lead to dysfunction of axons which contributes to neurodegeneration (Gendron and Petrucelli, 2009).

Interestingly, HDAC6 protein level has been shown to affect tau phosphorylation in cells. Decreased tau phosphorylation was observed in cells with knockdown of HDAC6 (Ding et al., 2008). In addition, inhibition HDAC6 by tubacin also attenuates tau phosphorylation, indicating catalytic activity of HDAC6 is also involved in this regulation (Ding et al., 2008). Therefore, augment of HDAC6 protein levels or catalytic activity may lead to hyperphosphorylated tau and subsequent tau aggregation which in turn contributes to thepathogenesis of AD. In support of this hypothesis, elevation of HDAC6 has been observed in AD brain (Ding et al., 2008). Nevertheless, more detailed study is needed to establish the casual relationship between increased HDAC6 and development of AD.

However, the effect of HDAC6 on tau is a multi-faceted case. Several researchers have shown that HDAC6 is involved in accumulation of tau into aggresome which initiates its turnover by autophagy (Ding et al., 2008, Guthrie and Kraemer, 2011). Moreover, formation of tau aggresomes prevents cytotoxicity of aggregated tau. In HDAC6 knockdown cells, abrogation of tau aggresome formation leads to significant elevation of insoluble tau (Guthrie and Kraemer, 2011). Tau itself inhibits both HDAC6 activity and HDAC6-dependent function in aggresome pathways (Perez et al., 2009). Therefore, further accumulation of tau could lead to deficiency in aggresome formation and autophagic clearance of other aberrant proteins. Thus, it would be interesting to postulate that substantial accumulation of aggregated tau during the later stage of disease could further prevent clearance of aberrant proteins which accelerates progression of disease. This also highlights the importance of HDAC6-dependent aggresome formation

of tau which probably occurs in the early stage of neurodegenerative diseases when there is little inhibition of HDAC6 by tau.

Interestingly, HDAC6 seems to regulate tau function and pathogenesis in neurodegenerative diseases at different levels. While HDAC6 activity is involved in aggregated tau clearance, inhibition of HDAC6 activity could compensate for microtubule defects induced by tau. As a microtubule-associated protein and tubulin deacetylase, inhibition of HDAC6 catalytic activity has been shown to stabilize microtubules (Matsuyama et al., 2002, Asthana et al., 2013). Loss of the microtubule-stabilizing function of tau has been implicated in neurodegenerative diseases, such as Alzheimer's disease (Alonso et al., 1994, Alonso et al., 1996). Therefore, inhibition of HDAC6 activity may be of therapeutic value in the treatment neurodegenerative diseases where the loss of tau' microtubule-stabilizing function is involved. This hypothesis is supported by a recent study using a *Drosophila* model. Either pharmacological inhibition or null mutation of HDAC6 rescues tau-induced microtubule defects by increasing tubulin acetylation (Xiong et al., 2013). Therefore, HDAC6 inhibitors could be considered as the potential treatments targeting neurodegenerative diseases.

HDAC6 and α-synuclein in Parkinson's Disease

Lewy bodies represent abnormal accumulation of protein aggregates in neurons of Parkinson diseased (PD) brain (Wakabayashi et al., 2007). Lewy bodies are primarily composed of fibrils formed by α-synuclein (Wakabayashi et al., 2007). Interestingly, immunohistochemistry experiments have shown that HDAC6 is highly concentrated within lewy bodies in brain sections from PD patients, indicating that HDAC6 may be

involved in its formation (Kawaguchi et al., 2003). Indeed, HDAC6 colocalizes with α-synuclein in aggresome-like bodies in cells upon treatment of PD-like neurodegeneration inducer MPP+ (Su et al., 2011). Either knockdown or pharmacological inhibition of HDAC6 leads to cytoplasmic distribution of α-synuclein bodies, suggesting an essential role of HDAC6 in α-synuclein aggregation (Su et al., 2011).

This hypothesis was further supported a study in a *Drosophila* PD model. In this study, it was found that HDAC6 directly interacts with oligomeric α-synuclein (Du et al., 2010). More importantly, a HDAC6 null mutation leads to decreased number of α-synuclein inclusion bodies. This in turn increases α-synuclein toxicity and locomotion dysfunction (Du et al., 2010). Conversely, HDAC6 overexpression results in decreased α-synuclein oligomers, indicating more α-synucleinis aggregated into inclusion bodies which is neuroprotective (Du et al., 2010). Therefore, the level of HDAC6 appears to be correlated with α-synuclein inclusion formation. Augment of HDAC6 may therefore be a promising therapeutic strategy to treat α-synuclein-related neurodegenerative diseases, such as PD.

HDAC6 and TDP-43 in TDP-43 Neurodegenerative Disease

TAR-DNA binding protein (TDP-43) can alsoform cytoplasmic inclusions and has been associated with a number of neurodegenerative diseases, including ALS and FTDL (Lagier-Tourenne et al., 2010). As discussed above, parkin-mediated ubiquitinated TDP-43 triggers its binding to HDAC6 leading to its cytoplasmic translocation (Hebron et al., 2013). Another connection between TDP-43 and HDAC6 is associated with RNA binding ability of TDP-43. As a DNA-binding protein, TDP-43 also has RNA-binding

domain and can mediate exon skipping of CFTR and APOA2 (Buratti et al., 2001, Mercado et al., 2005). Coincidently, TDP-43 specifically binds mRNA of HDAC6 (Fiesel et al., 2010), thusit may regulate HDAC6 protein levels post-transcriptionally. In support of this hypothesis, knockdown of TDP-43 in both neuronal and non-neuronal cells leads to decreased mRNA and expression levels of HDAC6 (Fiesel et al., 2011). Downregulation of HDAC6 by knockdown of TDP-43 has also been observed in a *Drosophila* model (Fiesel et al., 2010). In addition, this downregulation of HDAC6 affects both HDAC6-dependent aggresome formation and toxic aggregate clearance. Knockdown of TDP-43 in cells decreases inclusion formation of polyQ-expanded ataxin-3 and this deficiency is restored when exogenous HDAC6 is introduced (Fiesel et al., 2010).

TDP-43 is typically a nuclear localized protein. However, in several neurodegenerative diseases, such as ALS, TDP-43 is pathologically modified at its C-terminal leading to its cytosolic translocation and formation of cytosolic inclusion bodies (Pesiridis et al., 2009). The finding that knockdown of TDP-43 suppresses HDAC6-dependent aggresome formation may shed light on the pathogenesis of cytosolic TDP-43 in neurodegenerative diseases. Because translocation of TDP-43 from the nucleus to the cytosol may abolish binding of TDP-43 to HDAC6 mRNA. Consequently, this would lead to downregulation of HDAC6 expression accompanied by compromised aggresome formation and enhancend toxicity from protein aggregates which contributes to neurodegeneration.

Future Directions

Despite the fact that HDAC6's catalytic activity is required for aggresom formation involving a suite of different proteins, the exact role HDAC6 activity plays in this process remains unknown. Nevertheless, it is possible that HDAC6 activity may contribute to motor trafficking as it does regulate microtubule acetylation which has been associated with motor trafficking (Reed et al., 2006). However, multiple lines of evidence also show that pharmacological inhibition of HDAC6 leads to increased microtubule acetylation which enhances motor trafficking (Dompierre et al., 2007, Chen et al., 2010, Kim et al., 2012). Thus, these findings make this hypothesis questionable. Alternatively, it can be postulated that an unknown HDAC6 substrate may be involved in this process. Deacetylation of this unknown substrate by HDAC6 may be required for aggresome assembly. Future investigation is needed to examine these hypothesises.

The second unsolved question lies in the mechanism by which HDAC6 regulates recruitment of autophagic machinery to aggresomes. While both activity and presence of HDAC6 are clearly required for recruitment of autophagosomes to the MTOC, how autophagosomes are recognized by the HDAC6-dynein complex for their movement to aggresomes remains unknown. It is possible that HDAC6 may have a LIR-like domain that interacts with LC3, thus capturing and recruiting autophagosomes. Alternatively, it can also be postulated that HDAC6 may indirectly interact with autophagosomes through an adaptor protein with a LIR domain, such as p62 or NBR1 (Pankiv et al., 2007, Perera et al., 2011). In fact, I recently identified the direct interaction between HDAC6 and p62 (Yan et al., 2013). The interaction site on p62 was mapped at an undefined region

between ZZ domain and TRAF6 domain (Yan et al., 2013). The future study will need to investigate whether LIR of p62 recruits autophagosomes to HDAC6.

The third unanswered question lies in relationship between HDAC6 and p62. It is obvious that these two proteins may play cooperative roles in targeting damaged mitochondria for their perinuclear accumulation and autophagic clearance. However, it remains unclear whether HDAC6 and p62 work together to regulate protein aggregation and their autophagic clearance. Interestingly, a recent study revealed that both HDAC6 and p62 are associated with aggregates formed by Toll-like receptor adaptor molecule MyD88 and are involved in formation of these aggregates (Into et al., 2010). While this observation supports the hypothesis that HDAC6 and p62 may work synergistically in protein aggregation and aggresome formation, the exact mechanism is yet to be investigated. The roles of p62 in protein aggregation and autophagic clearance have,however, been well characterized (Seibenhener et al., 2004, Bjorkoy et al., 2005, Pankiv et al., 2007). Thus, it is also interesting to postulate that p62 and HDAC6 may be functionally redundant in regulation the certain step in aggresome –autophagy pathway. If this is the case, it is possible to examine this hypothesis by studying either p62 function in a HDAC6 knockout model or HDAC6 function in a p62 knockout model as they relate to changes in the aggresome-autophagy pathway.

Finally, both *in vitro* and *in vivo* studies support the essential role of HDAC6 in inclusion body and lewy body formation and their autophagic clearance in neurodegenerative diseases (Kawaguchi et al., 2003, Iwata et al., 2005b). These findings thus open the possibility of augmenting HDAC6 as a promising therapeutic strategy to attenuate neurodegenerative diseases. However, several *in vitro* studies have

demonstrated that pharmacological inhibition of HDAC6 leads to increased microtubule acetylation and enhanced motor-mediated trafficking of BDNF and mitochondria in neurons (Dompierre et al., 2007, Chen et al., 2010, Kim et al., 2012). In addition, inhibition of HDAC6 has also been shown to reverse axonal transport deficit in a mouse model of neurodegenerative disease (d'Ydewalle et al., 2011). Therefore, inhibition of HDAC6 may also be of therapeutic value to treat neurodegenerative diseases. While this seems to be contradictory to the strategy mentioned above, the discrepancy may be due to the different pathological features in distinctive neurodegenerative disease models used in these studies. Therefore, both strategies need to be applied with caution as attenuation of one deficit may result in alleviation of the other deficit during the progression of the disease.

Concluding Remarks

In summary, current research advances have shown that HDAC6 and its interacting partners are essential components for the efficient processing of protein aggregates and damaged mitochondria in the aggresome-autophagy pathway. The interplay between HDAC6 and its interacting partners are critical for maintaining functionality of these degradation pathways which are in turn critical for removal of aggregate-prone proteins as found associated with neurodegenerative disease. Therefore, future research would deepen our understanding of mechanisms by which HDAC6 and its interacting partners regulate the aggresome-autophagy pathway, thus facilitating the development of therapeutic strategy to treat neurodegenerative diseases.

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CHAPTER II. GENENERAL INTRODUCTION: STRUCTURE AND FUNCTIONS OF SQSTM1/P62 AND HDAC6

Structure and functions of p62

Sequestosome 1 (SQTM1)/ p62 was initially identified as an interacting protein for atypical protein kinase C (aPKC) (Sanchez et al., 1998). It has several protein-interacting domains and thus is linked to various cell signaling cascades. p62 serves as a shuttling factor in ubiquitin-proteasome system. With binding of polyubiquitinated proteins through its C-terminal UBA domain, p62 can recruit ubiquitin-tagged misfolded proteins (Seibenhener et al., 2004, Babu et al., 2005) (Fig. 1-1). These misfolded proteins are targeted to proteasome by the interaction between p62's N-terminal PB1 domain and 19S proteasome component Rpt1 (Seibenhener et al., 2004) (Fig. 1-1). Interestingly, p62 also plays a role in an alternative degradation pathway autophagy. P62 has a LIR that directly interacts with LC3, mammalian homologue of yeast autophagy gene ATG8 (Pankiv et al., 2007). As LC3 is a crucial component of autophagosome, p62 serves as a bridge to recruit polyubiquitinated proteins to autophagosome for their autophagic degradation (Pankiv et al., 2007) (Fig. 1-1). The ZZ domain of p62, found close to its PB1 domain, is the ZZ-type of zinc finger motif that binds death receptor interacting protein RIP1 to activate NF-κB and p38MAPK signaling pathways (Sanz et al., 1999, Kawai et al., 2008) (Fig. 1-1). ZZ domain is also associated with receptor

trafficking to synaptic plasticity through interaction with AMPA receptor subunit, GluR1-3 (Jiang et al., 2009). For this regulation, p62 scaffolds aPKC phosphorylation on GluR1 which is critical for trafficking of receptor to surface (Jiang et al., 2009) (Fig. 1-1) In addition, p62 also can regulate internalization of neurotrophin receptor TrkA by recruiting E3 ubiquitin ligase TRAF6 through its TRAF6 binding domain (Geetha et al., 2005). P62 serves as a scaffold for K63-polyubiquitination of TrkA by TRAF6 which directs internalization of this neurotrophin receptor (Geetha et al., 2005) (Fig. 1-1).

Figure 1-1

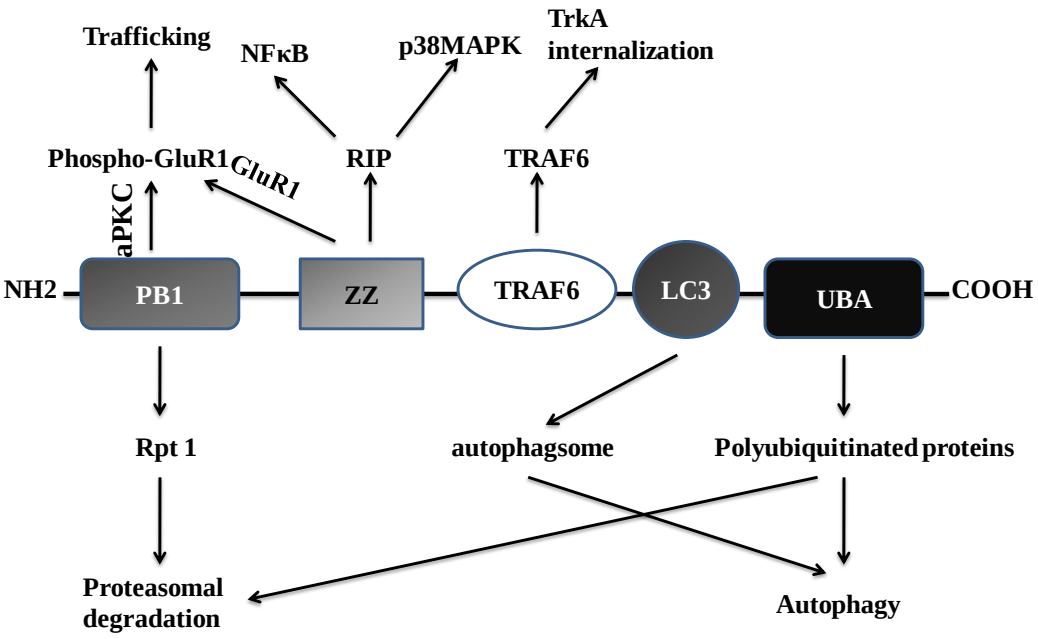

Figure 1-1 **Domain structure and functions of p62.** Please reference the text.

An important function of p62 is regulation of protein trafficking. Past work in our laboratory revealed that p62 regulates neurotrophin receptor TrkA trafficking (Geetha and Wooten, 2003). In addition, p62 is also critical for trafficking of protein aggregates to a cellular collection center, termed aggresomes or inclusion bodies (Nagaoka et al., 2004, Seibenhener et al., 2004, Pankiv et al., 2007). This trafficking is dependent on movement of motor proteins along microtubule railway track (Kopito, 2000). Knockdown of p62 in cells significantly reduces formation of aggresome-like inclusions, indicating a trafficking deficiency (Pankiv et al., 2007). The mechanism by which p62 regulates trafficking of protein aggregates to aggresomes remains unclear. P62 can bind polyubiquitinated protein aggregates through its UBA domain (Layfield et al., 2004). Thus, it is possible that p62 may be important for loading protein aggregates to motor protein for microtubule-dependent trafficking. Alternatively, it is also possible that p62 can affect binding affinity and progressivity of motor protein on microtubules. Either hypothesis is yet to be investigated.

Structure and functions of HDAC6

Interestingly, studies in recent years have shown that binding affinity and progressivity of motor protein on microtubules are regulated by the acetylation state of tubulin subunits (Reed et al., 2006, Dompierre et al., 2007). Tubulin acetylation occurs on Lysine 43 of α-tubulin (Nogales et al., 1998, Nogales et al., 1999). It is a reversible reaction controlled by two classes of enzymes, namely acetytransferases and deacetylases. So far, only two deacetylases have been identified that deacetylate tubulin. The human

homolog of yeast silent information regulator 2 protein (Sir2p), sirtuin type 2 (SIRT2), was identified as a NAD+ dependent tubulin deacetylase (North et al., 2003). Another tubulin deacetylase is histone deacetylase 6 (HDAC6) (Hubbert et al., 2002). It belongs to class II histone deacetylases. The predominant cytoplasmic localization of HDAC6 allows it to deacetylate cytoplasmic proteins, distinct from the rest of HDACs (Verdel et al., 2000). Besides α-tubulin, Hsp90 and cortactin were also identified as HDAC6 cytoplasmic substrates (Kovacs et al., 2005, Zhang et al., 2007) . HDAC6 has two independent deacetylase domains, termed DD1 and DD2 (Zhang et al., 2006), both of which are involved in deacetylase activity (Hubbert et al., 2002). DD2 of HDAC6 has been shown to be specifically responsible for tubulin deacetylase activity (Haggarty et al., 2003). Therefore, HDAC6 plays an important role in regulation of tubulin acetylation. Both genetic inactivation of HDAC6 and pharmacological inhibition of HDAC6 results in increased acetylated tubulin (Zhang et al., 2003). Inhibition of HDAC6 indeed enhances motor trafficking on microtubules by elevating tubulin acetylation (Dompierre et al., 2007, Chen et al., 2010).

HDAC6 is also an essential component of the aggresome-autophagy pathway. HDAC6 serves as an adaptor by binding ubiquitinated proteins and dynein motor through its C-terminal BUZ domain and dynein binding domain (DMB) respectively, during the retrograde transport of these proteins to the MTOC leading to the formation of aggresomes (Kawaguchi et al., 2003). Interestingly, HDAC6 activity is also required for this process (Kawaguchi et al., 2003). HDAC6 also regulates autophagosome-lysosome fusion by stimulating cortactin-dependent actin remodeling. In this regulation event, deacetylationof cortactin by HDAC6 is required to induce actin remodeling (Lee et al.,

2010). Besides its critical roles in the aggresome-autophagy pathway, HDAC6 also regulates microtubule dynamic properties as a microtubule-associated protein and tubulin deacetylase. Overexpression of HDAC6 has been shown to decrease resistance of microtubules to depolymerizing drug (Matsuyama et al., 2002) while depletion of HDAC6 enhances microtubule stability (Tran et al., 2007). In addition, activity of HDAC6 has been associated with microtubule assembly rate. Pharmacological inhibition of HDAC6 in cells has been shown to reduce microtubule growth rate or delay microtubule reassembly (Zilberman et al., 2009, Asthana et al., 2013).

Proposal

Based on the existing understanding of the functionality of both p62 and HDAC6, I initially proposed a model whereby p62 might affect microtubule-dependent trafficking of protein aggregates through its connection with HDAC6. To investigate this hypothesis, I first sought to identify and characterize the possible interactions between p62 and HDAC6. Based on the positive results from this work, I then moved to study how this interaction affects HDAC6 function, as well as, processing of protein aggregates within the aggresome-autophagy pathway. Since HDAC6 function is also associated with microtubule dynamic properties, I included in my dissertation research a study to determine whether p62 could regulate microtubule dynamics through modulation of HDAC6.

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*[1]CHAPTER III.SQSTM1/p62 Interacts with HDAC6 and Regulates Deacetylase Activity

Abstract

Protein aggregates can form in the cytoplasm of the cell and are accumulated at aggresomes localized to the microtubule organizing center (MTOC) where they are subsequently degraded by autophagy. In this process, aggregates are engulfed into autophagosomes which subsequently fuse with lysosomes for protein degradation. A member of the class II histone deacetylase family, histone deacetylase 6 (HDAC6) has been shown to be involved in both aggresome formation and the fusion of autophagosomes with lysosomes making it an attractive target to regulate protein aggregation. The scaffolding protein sequestosome 1(SQSTM1)/p62 has also been shown to regulate accumulation and autophagic clearance of protein aggregates. Recent studies have revealed colocalization of HDAC6 and p62 to ubiquitinated mitochondria, as well as, ubiquitinated protein aggregates associated with the E3 ubiquitin ligase TRIM50. HDAC6 deacetylase activity is required for aggresome formation and can be regulated by protein interaction with HDAC6. Due to their colocalization at ubiquitinated protein aggregates, I sought to examine if p62 specifically interacted with HDAC6 and if so, if this interaction had any effect on HDAC6 activity and /or the physiological function of cortactin-F-actin assembly. I succeeded in identifying and mapping the direct interaction

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between HDAC6 and p62. I further show that this interaction regulates HDAC6 deacetylase activity. Data are presented demonstrating that the absence of p62 results in hyperactivation of HDAC6 and deacetylation of α-tubulin and cortactin. Further, uponinduction of protein misfolding I show that p62 is required for perinuclear co-localization of cortactin-F-actin assemblies. Thus, these findings indicate that p62 plays a key role in regulating the recruitment of F-actin network assemblies to the MTOC, a critical cellular function that is required for successful autophagic clearance of protein aggregates.

Introduction

Misfolded proteins are thought to be sequestered into aggregates for the protection of cells as accumulation of mis-functional proteins can be toxic (Goldberg, 2003). This process was originally attributed to ubiquitin tagging of defective proteins leading to their recruitment into aggresomes that are degraded by autophagy (aggresome-autophagy pathway) (Yao, 2010). However, recent advancements have shown that protein recruitment can also occur in an ubiquitin-independent manner (Watanabe and Tanaka, 2011). The Class II histone deacetylase HDAC6 has been associated with aggresome formation in both ubiquitin dependent (Kawaguchi et al., 2003, Iwata et al., 2005b) and independent pathways (Watanabe and Tanaka, 2011) suggesting HDAC6 may play a pivotal role in both protein accumulation and cell protection. HDAC6 is predominantly localized to the cytoplasm, a feature that distinguishes it from other HDAC family members (Verdel et al., 2000). HDAC6 contains two catalytic domains, DD1 and DD2 (Hubbert et al., 2002), as well as, a C-terminal ubiquitin binding

domainBUZ/ZnF-UBP (Seigneurin-Berny et al., 2001, Kawaguchi et al., 2003, Boyault et al., 2006). Polyubiquitinated protein aggregates are recruited to HDAC6 through this BUZ domain (Kawaguchi et al., 2003, Olzmann et al., 2007), while deacetylase activity is regulated by one or both of the internal catalytic domains (Hubbert et al., 2002, Zhang et al., 2006). It has been proposed that HDAC6 facilitates loading of aggregated proteins onto the dynein motor protein complex by serving as an adaptor between ubiquitinated protein aggregates and dynein (Kawaguchi et al., 2003). As such, a functional interaction exists between HDAC6, the motor protein dynein, and polyubiquitinated proteins in aggresome formation at the microtubule organizing center (MTOC) (Kawaguchi et al., 2003). Knockdown of HDAC6 results in impairment of polyubiquitinated proteins recruitment to dynein and subsequent transport to the MTOC leading to an aggresome-deficient phenotype (Kawaguchi et al., 2003). Interestingly, the role of HDAC6 in the aggresome-autophagy pathway is not solely that of an adaptor protein as deacetylation of its substrate cortactin is required for autophagosome-lysosome fusion (Kawaguchi et al., 2003, Lee et al., 2010a). Thus, both accumulation of protein aggregates at aggresomes and their autophagic clearance occur in an HDAC6-dependent fashion.

A number of proteins have been found to regulate the activity of HDAC6. Both epidermal growth factor receptor (EGFR) (Deribe et al., 2009) and casein kinase 2 (CK2) (Watabe and Nakaki, 2011) regulate HDAC6 activity by phosphorylation, leading to changes in cellular acetylated tubulin levels. Expression of a CK2 phosphorylation site mutant of HDAC6(S458A) has been shown to abrogate recruitment of the HDAC6 substrate cortactin to aggresomes (Watabe and Nakaki, 2011). Failure of this recruitment leads to inability of the associated F-actin assembly network to organize properly which

subsequently results in failure to clear aggregated proteins (Lee et al., 2010a). Other proteins, such as dysferlin, can also regulate HDAC6 deacetylation of tubulin by interfering with the interaction between HDAC6 and tubulin itself (Di Fulvio et al., 2011). In addition, the HDAC6-interacting protein tau has been shown to inhibit HDAC6 deacetylase activity with overexpression of tau leading to inhibition of aggresome formation (Perez et al., 2009).

Interestingly, HDAC6 has recently been shown to also be involved in mito-aggresome formation that is associated with elimination of damaged mitochondria(Lee et al., 2010b). In this process, that closely resembles aggresome formation, the atypical protein kinase C (aPKC)-interacting protein sequestosome 1/p62 (hereafter referred to as simply p62) has been reported to co-localize with HDAC6 in ubiquitinated mito-aggresomes (Lee et al., 2010b). p62 has been found to have myriad roles in cellular mechanics, not the least of which is a well-defined function in intracellular disposal pathways. In this role, p62 is involved in transport of both misfolded proteins and dysfunctional organelles to cellular degradation sites (Seibenhener et al., 2004, Okatsu et al., 2010). This transport is accomplished via the microtubule network where the motor protein dynein"moves" cargoes along the microtubule concentrating damaged proteins and organelles into aggresomes or inclusion bodies (Johnston et al., 1998, Kopito, 2000, Lee et al., 2010b). Of particular importance in this role is the presence in p62 of a C-terminal UBA domain for binding of ubiquitin and ubiquitinated protein aggregates. Recent studies have shown p62 is involved in inclusion body formation and selective autophagic clearance of ubiquitinated substrates (Bjorkoy et al., 2005, Pankiv et al., 2007, Ichimura et al., 2008). In association with mitochondrial clearance by mitophagy, both

p62 and HDAC6 are recruited to mitochondria ubiquitinated by parkin (Lee et al., 2010b). Similarly, both proteins have also been shown to interact with the E3 ubiquitin ligase TRIM50 (Fusco et al., 2012) localizing to aggregate formation sites where they promote the sequestration and clearance of ubiquitinated proteins at aggresomes.

Previous work in our laboratory has documented that loss of p62 abrogates movement of protein aggregates and organelles (Seibenhener et al., 2004, Seibenhener et al., 2013). Because both p62 and HDAC6 are known to be closely associated with aggregate clearance and both proteins show co-localization, I reasoned that p62 might directly or indirectly affect the activity of HDAC6.To test this hypothesis, we examined tubulin acetylation in a p62 knock-out model. My goal was to determine what, if any, effect the presence of p62 has on the deacetylase activity of HDAC6 and how this might relate to our previous observation of impaired motor transport. In the results reported here, I identify a specific binding domain of p62 which does interacts with a catalytic domain of HDAC6 resulting in modulation of HDAC6 deacetylase activity. I show that lack of p62 hyper-activates HDAC6 resulting in elevated de-acetylation of the HDAC6 specific substrates α-tubulin and cortactin. I also reveal that elevation of HDAC6 activity by loss of p62 leads to an increased association of F-actin network assemblies with aggregates containing HDAC6 which are unable to move to the MTOC for autophagic degradation.

Materials and Methods

Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells from the American Type Culture Collection were grown as described previously (Wooten et al., 2005). Transfection was

achieved using the Mammalian Cell Transfection Kit (EMD-Millipore, Billerica, MA). Wild Type (WT) and p62 knock-out (p62KO) mouse embryonic fibroblasts (MEF) were derived from E13.5 mouse embryos (Duran et al., 2004) and grown in DMEM supplemented with 10% Fetal Calf Serum and antibiotics at 37^{o}C with high humidity and 5%CO_2. Transfection of MEF cells was carried out using Lipofectamine 2000 (Life Technologies, Grand Island, NY).

Antibodies, Reagents, and Plasmid Constructs

Monoclonal antibodies for α-tubulin, acetylated α-tubulin and FLAG-tag were obtained from Sigma Chemical (St. Louis, MO). p62 monoclonal antibody was purchased from Abcam (Cambridge, MA). All other antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). All reagents were purchased from Sigma Chemical (St. Louis, MO). Tubacin and nil-Tubacin were a generous gift from Dr. Stuart Schreiber, Harvard University. WT and deletion mutants of p62 constructs were generally provided by Dr. Jorge Moscat. Truncation mutants of HA-HDAC6 constructs were gifts from Wooten lab's collaborator.

Immunoprecipitation and Western Blot

Either HEK or MEF cells were lysed on ice using Triton Lysis Buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 10mM NaF, 0.5% Trition X-100, 1mM Na_3VO_4, 1mM PMSF, 2μg/ml aprotinin and leupeptin). Protein concentration was determined by Bradford Assay (Bio-Rad, Hercules, CA) prior to immunoprecipitation. Lysates were rotated overnight at 4^{o}C with primary antibody followed by 3 hours with anti-IgG-

agarose beads. Precipitates were washed with Triton Lysis Buffer a total of 3 times prior to the addition of 1X Sample Buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose for Western blotting.

GST-Pulldown Assay

E. coli cells expressing GST-p62 were grown in 2xYT media (16g tryptone, 10g yeast extract, 5g NaCl, 0.49g sodium citrate, 6.27g K_2HPO_4, 1.63g KH_2PO_4per 1 liter, pH 7.6 supplemented with 100mM $MgSO_4$ and antibiotic) for 12 hours. Expression was induced with 1mM IPTG for 4 hrs. Bacterial cells were lysed with NETN buffer (20mM Tris, pH 8.0, 100mM $NaCl_2$, 1mM EDTA, 0.1% NP40, 2µg/ml leupeptin, 1mM PMSF). GST-p62 was purified from bacterial lysates by binding to glutathione-agarose beads overnight at 4^{o}C. Bound beads were then washed 5 times with NETN followed by resuspension in NETN. Protein concentration was determined by Bradford Assay.

HEK cell lysates expressing HA-HDAC6 were added to 10µg GST-p62 beads and allowed to bind overnight at 4^{o}C. Following incubation, beads were washed 3 times with NETN buffer and 1X Sample Buffer added. Pulldown samples were separated on SDS-PAGE followed by Western blot.

HDAC6 Activity Assay

HDAC6 was purified from WT and p62KO MEF cells by immunoprecipitation. HDAC activity was then measured using the HDAC Colorimetric Activity Assay kit (Biovision, Milpitas, CA). Briefly, HDAC6 immunoprecipitates were incubated with a HDAC colorimetric substrate consisting of polypeptide chains with acetylated lysine side

chains. Following incubation per the manufacturer's instructions, the reaction was stopped by incubation with developer. Colorimetric detection was determined at 405nm.

Immunofluorescence Microscopy

WT and p62KO MEF cells were fixed in 4% paraformaldehyde for 1 hour. Cells were then washed with PBS prior to permeabilization with0.1% TX-100/PBS for 10 minutes. After permeabilization, cells were blocked in 3% milk/PBS at room temperature for 4 hours before adding primary antibodies in blocking solution. Fluorescently tagged secondary antibodies (Life Technologies, Grand Island, NY) were added in block for 2 hours at room temperature. Actin was stained using AF350-phalloidin (Life Technologies, Grand Island, NY). Colocalization of proteins was visualized using a Nikon A1/T1 confocal microscope and the Nikon Elements software.

Statistical Analysis

Means, standard errors, Student's *t* tests and Chi-squared tests were calculated manually (Sokal and Rohlf, 2005). Chi-squared statistical were calculated under the assumption of unequal group variances with one-tailed p-values. P-values less than 0.05 were considered significant.

Results

p62 affects acetylated tubulin levels in mouse embryonic fibroblasts

My first objective was to determine the activity level of HDAC6 in the absence of p62. For this experiment, I sought to examine tubulin acetylation levels in WT and

p62KO MEF cells via Western blot with acetyl-tubulin specific antibody. While α-tubulin levels remained relatively constant between WT and p62KO cells, I observed a significant decrease in acetylation of tubulin in the absence of p62 (WT vs. p62KO: $t(4)=29.03$, $p=4.19E-6$) (Fig. 3-1A). Deacetylation of tubulin can be accomplished by the HDAC family of deacetylases (North et al., 2003, Zhang et al., 2003). Upon treatment with the general class I and II HDAC inhibitor Trichostatin A (TSA), deacetylation of tubulin was inhibited while treatment with nicotinamide, a class III NAD+- dependent SIRT family inhibitor, did not inhibit deacetylation of tubulin. α-Tubulin is a specific substrate of HDAC6 (Matsuyama et al., 2002, Haggarty et al., 2003, Zhang et al., 2003). In WT or p62KO cells, treated with the HDAC6 specific inhibitor tubacin, deacetylation of tubulin was inhibited while treatment with the inactive analog, nil-tubacin produced no evidence of deacetylation inhibition. Examination of the ultra-structure of tubulin filaments using immunofluorescence-based microscopy showed characteristic acetylated tubulin staining along filaments in WT cells but disorganized and diffuse acetyl-tubulin staining in cells lacking p62 (Fig. 3-1B). I concluded from these results that p62 could play a regulatory role in the deacetylase activity of the HDAC family member HDAC6.

p62 interacts with the HDAC family member HDAC6

In general, regulatory proteins interact directly with their substrates. To date, a number of proteins have been identified that regulate HDAC6 activity through physical interaction (Perez et al., 2009, Wickstrom et al., 2010, Di Fulvio et al., 2011). Thus, Ihypothesized that the regulatory effect of p62 on the deacetylation activity of HDAC6 could be accomplished by direct interaction between p62 and HDAC6. Using bacterially

expressed GST-tagged p62, a pulldown experiment incorporating exogenous HA-tagged HDAC6 protein was performed. Results from this experiment provided strong evidence of a direct interaction between p62 and HDAC6 in an *in vitro* environment (Fig. 3-2A). Interaction between the two proteins was further confirmed by co-precipitation using exogenously expressed constructs (Fig. 3-2B), as well as, endogenously expressed proteins (Fig. 3-2C) indicating interaction between p62 and HDAC6 occurred *in vivo* as well as *in vitro*. Evidence of endogenous p62 and HDAC6 co-localization was also observed when WT MEF cells were examined by immunofluorescence staining and confocal microscopy (Fig. 3-2D).

Mapping the interaction sites between p62 and HDAC6

As a highly specific interaction was observed between p62 and HDAC6 (Fig. 3-2A-D), I next sought to determine the interaction domains of both proteins using deletion constructs. A full length tagged-p62 construct along with various internal domain deletions (Fig. 3-3A) were expressed in HEK cells with FLAG-HDAC6. Co-immunoprecipitation was performed using a FLAG-antibody for HDAC6 and Western blots were generated to the tagged-p62 deletion constructs. p62 interaction was seen for all constructs with the exception of the p62 (Δ1-229) which encompassed only the carboxyl terminal half of the protein (Fig. 3-3B). When alignment of all constructs was performed, the HDAC6 binding region was localized to residues 164-225, the undefined region between p62's ZZ domain and the identified TRAF6 binding region (shaded area of Fig. 3-3A).

To map the region of HDAC6 interacting with p62, tagged HDAC6 truncation constructs (Fig 3-4A) were expressed in HEK cells along with myc-tagged full length p62 and co-immunoprecipitation performed as above. HDAC6's catalytic domains DD1 and DD2 have been shown to play critical roles in the protein's catalytic activity (Zhang et al., 2006). In particular, DD2 was indicated to specifically regulate the deacetylase activity of HDAC6 and to possess a catalytic site inhibited by tubacin (Haggarty et al., 2003). Of the truncated HDAC6 constructs examined, only those containing the DD2 domain showed interaction with p62 (Fig. 3-4B). When alignment of the constructs was performed, the p62 binding domain was localized to residues 429-824 which encompass the entirety of the DD2 domain (shaded area of Fig. 3-4A). Collectively, these experiments confirm the direct interaction between p62 and HDAC6 and map specific interaction domain within both proteins.

p62 inhibits the deacetylase activity of HDAC6

I have shown evidence for a specific interaction between p62 and HDAC6 that correlates with increased HDAC6 specific deacetylation of an *in vivo* substrate, α-tubulin, in the absence of p62. Based on these results, we next sought to examine if the increase in HDAC6 activity was specific to α-tubulin or a generalized catalytic increase caused by p62 driven deregulation. HDAC6 was immunoprecipitated from WT and p62KO MEF cells and activity of the immune complex was determined by *in vitro* deacetylation of a commercial acetylated substrate (Fig. 3-5A). HDAC6 immunoprecipitated from p62KO cells showed significantly increased deacetylase activity using this *in vitro* substrate. As p62 does appear to regulate HDAC6 deacetylase activity on both *in vivo* and *in vitro*

substrates, we reasoned that return of p62 to the p62KO null background would reestablish control of HDAC6 activity. Exogenous p62 was transfected into p62KO MEF cells and tubulin acetylation examined by Western blot (Fig.3- 5B). Acetylated tubulin levels were increased in transfected cells and, while not completely reaching WT acetylation thresholds, reestablishment of p62 regulated the specific HDAC6 deacetylation of tubulin.

p62 is required for the physiological function of the cortactin-F-Actin assembly

As p62 was found to regulate HDAC6 activity on both *in vitro* and *in vivo* substrates, I next sought to examine its effect on HDAC6 activity in a physiological process. HDAC6 has been shown to be integral for the recruitment of cortactin (Zhang et al., 2007), along with F-actin assemblies to perinuclear protein aggregates (Lee et al., 2010a). Once recruited, cortactin is deacetylated by active HDAC6 leading to autophagosome-lysosome fusion and protein aggregate clearance (Lee et al., 2010a). I reasoned that if HDAC6 activity is negatively regulated by the presence p62, cortactin acetylation levels in p62KO cells could be decreased. When cortactin was immunoprecipitated from either WT or p62KO MEF cells, acetylated cortactin levels were significantly decreased in the absence of p62 (Fig. 3-5C) further supporting a regulatory role for p62 in the activity of HDAC6.

F-actin remodeling is required for quality control (QC) autophagy-dependent degradation of protein aggregates and deacetylation of cortactin is critical to this process (Lee et al., 2010a). In HDAC6 KO MEF cells, loss of HDAC6 prevents the co-localization of cortactin/F-actin with protein aggregates in an activity dependent

fashion(Lee et al., 2010a). As I have shown that p62 plays a role in regulating HDAC6 activity on cortactin deacetylation, I sought to determine what role, if any, p62 plays in cortactin/F-actin assembly. We examined cortactin/HDAC6 colocalization at sites of F-actin assemblies in WT and p62KO MEF cells by immunofluorescence (Fig. 3-6) and quantitatively estimated Mander's Overlap Coefficient values of colocalization (Zinchuk et al., 2007) using the NIS Elements software (Nikon). Evidence of recruitment of cortactin to F-actin was seen in WT and p62KO cells as noted by colocalization of actin and cortactin (Fig. 3-6A d – Mander's = 0.877; Fig. 6B d – Mander's = 0.729). However, co-localization of HDAC6 and cortactin was more evident in p62KO MEFS (Fig. 3-6B f – Mander's = 0.968) compared to WT (Fig. 6A f – Mander's = 0.864) as would be expected if HDAC6 activity is deregulated by the absence of p62. If this is indeed the case, increased HDAC6 activity would cause deacetylation of cortactin leading to increased cortactin/F-actin association with aggregates (Fig. 3-6A vs Fig. 3-6B merge for non-treated cells).

To induce QC-autophagy, cells were treated with MG132 to promote protein misfolding. I observed an overall increase in HDAC6-cortactin co-localization in the induced samples for WT(Fig. 3-6A f – Mander's = 0.864 vs. Fig. 6A l – Mander's = 0.987). However, untreated p62KO cells showed high levels of co-localization between HDAC6 and cortactin that were unresponsive to MG132 treatment (Fig. 3-6B f – Mander's = 0.968 vs. Fig. 6B l – Mander's = 0.971). This result is indicative of higher *in vivo*stress levels due to the lack of p62 in these cells (Ramesh Babu et al., 2008). Importantly, induced HDAC6-cortactin / F-actin assemblies in WT cells were predominately found in the perinuclear region of the cell as would be expected in a

normally functioning cell. Conversely, these assemblies remained localized in the cytosol in the absence of p62 (Fig. 3-6A vs3-6B, merge for MG132-treated cells). Therefore, I concluded that while recruitment of cortactin to F-actin is unaffected in the absence of p62 (Fig. 3-6A k; Fig. 3-6B k), F-actin remodeling is abrogated by the lack of p62 (Fig. 3-6B merge for MG132-treated cells).

Discussion

HDAC6 is one of the most extensively studied members of the histone deacetylase family of proteins (Sadoul et al., 2011). HDAC6 is exclusively localized in the cell cytoplasm and has a number of cytoplasmic substrates including α-tubulin (Hubbert et al., 2002), cortactin (Zhang et al., 2007), HSP90 (Kovacs et al., 2005) and peroxiredoxin (Parmigiani et al., 2008). Cytoplasmic localization and the functions of its substrates implicate HDAC6 in a number of cellular regulatory processes. Because of its involvement in multiple activities, how HDAC6 is regulated has become an area of intense interest.

As is true for many essential proteins, HDAC6 appears to be regulated at multiple levels. One observed mode of HDAC6 regulation is specifically associated with a change in its localization within the cytoplasm. HDAC6 can be translocated by association with its substrate HSP90, along with Rac1, to membrane ruffles after PDGF stimulation where it can influence actin dynamics (Gao et al., 2007) resulting in cell migration. Another mode of HDAC6 activity regulation is via post translational modification such as phosphorylation. HDAC6 is phosphorylated by the EGFR after ligand-induced receptor binding leading to increased acetylated tubulin and delivery of endocytosed EFGR to the

lysosome for degradation (Deribe et al., 2009). GSK3β-dependent phosphorylation may also enhance the activity of HDAC6 leading to decreased tubulin acetylation and an inhibition in mitochondrial motility (Chen et al., 2010).

Yet a third method of HDAC6 regulation is the direct or indirect binding of various partners to HDAC6 itself. A complex composed of HDAC6, farnesyltransferase and microtubules can be essential for HDAC6 activity. Disruption of this complex by inhibition of the transferase activity results in increased tubulin acetylation (Zhou et al., 2009). Similarly, by direct binding to tau, the deacetylase activity of HDAC6 is inhibited resulting in increased tubulin acetylation (Perez et al., 2009). An excess of tau protein may act as an HDAC6 inhibitor preventing autophagy induced by proteasome inhibition. Thus, tau has been shown to not only directly inhibit the deacetylase activity of HDAC6, but also impair HDAC6-dependent autophagy (Perez et al., 2009).

I have shown here that the scaffolding protein p62 can directly bind to one of the two catalytic domains of HDAC6 and that p62 can regulate the deacetylase activity of HDAC6. As the binding site for direct p62 interaction with HDAC6 is located in the catalytic DD2 domain, it is possible that this direct interaction is responsible for HDAC6 inhibition. As HDAC6 requires both DD1 and DD2 catalytic domains to function (Zhang et al., 2006), physical interaction of p62 with one of these domains could inhibit HDAC6 deacetylase activity. However, p62 was first recognized as an aPKC-interacting protein where it supports the phosphorylation activity of aPKC (Puls et al., 1997). It is therefore possible that the interaction of p62 with HDAC6 provides a scaffold for the recruitment of aPKC. As discussed above, phosphorylation can lead to change of HDAC6 activity

(Deribe et al., 2009, Chen et al., 2010). Once present, aPKC could phosphorylate and alter HDAC6 activity. In fact, we have shown that HDAC6, p62 and aPKC do exist in a ternary complex (data not shown). However, the physiological role of aPKC on HDAC6 phosphorylation and subsequent activation has not been elucidated. My data did clearly show that a specific and direct interaction between p62 and HDAC6 regulates the deacetylase activity of HDAC6 towards its substrates.

α-tubulin together with α-tubulin form the heterodimeric building blocks of microtubules (MacRae, 1992). Acetylation of α-tubulin at Lys40 is thought to stabilize microtubules in the cell (LeDizet and Piperno, 1987, Piperno et al., 1987). This line of thought is somewhat controversial as to whether acetylation is the cause or consequence of stabilization (Zhou et al., 2004). Regardless, acetylation of α-tubulin is a characteristic of stable microtubules (Rosenbaum, 2000, Westermann and Weber, 2003) along with tau interaction (Lee and Rook, 1992). Destabilization of microtubules by deacetylation or drug treatment has been linked to neurodegenerative diseases (Li et al., 2011). I have previously shown that p62KO mice display characteristics biochemically and behaviorally similar to an Alzheimer's disease mouse model (Ramesh Babu et al., 2008). Thus, dysregulation of HDAC6 activity by loss of p62 could affect the stability of microtubules leading to neurodegenerative disease.

In addition to its role in stabilization of microtubules, tubulin acetylation also plays a role in enhancing protein trafficking along microtubules in polarized cells (Reed et al., 2006). Tubulin acetylation enhances recruitment of the molecular motors kinesin and dynein to microtubules to promote vesicular transport (Reed et al., 2006, Dompierre

et al., 2007). In fact, kinesin binding and transport of the cargo protein Jip-1(Reed et al., 2006), as well as kinesin bound mitochondria in neurons (Chen et al., 2010),are enhanced by tubulin acetylation. Increased HDAC6 activity could disrupt trafficking in the cell by removing acetyl groups from α-tubulin implicating p62 not only in the formation of protein aggregates but also in the regulation of their transport to the processing centers of the cell.

HDAC6 has emerged as an important player in the regulation of cellular protein aggregates due to its cytoplasmic localization, its association with the microtubule transport network and its ability to associate with ubiquitinated substrates via its C-terminal BUZ/ZnF-UBP domain (Seigneurin-Berny et al., 2001, Kawaguchi et al., 2003, Boyault et al., 2006). HDAC6 has been recognized as a main regulatory component of the aggresome, the MTOC localized inclusion body where excess protein aggregates are trafficked and disposed (Kawaguchi et al., 2003). HDAC6 has also been implicated in removal of aggresomes by QC-autophagy linking HDAC6 to this cellular clearance process as well (Iwata et al., 2005b, Pandey et al., 2007, Lee et al., 2010a). HDAC6 is part of the F-actin remodeling machinery where it facilitates the fusion of autophagosomes with lysosomes leading to autophagic clearance (Lee et al., 2010a). HDAC6 deacetylates cortactin as part of this complex, promoting F-actin remodeling leading to fusion of the autophagosome to lysosomes and protein aggregate clearance at the aggresome (Lee et al., 2010a).

p62 is also implicated in aggregate clearance by its ability to bind to both the autophagic marker protein LC3 and ubiquitinated substrates via its C-terminal UBA

domain leading to protein aggregate clearance (Bjorkoy et al., 2005, Pankiv et al., 2007). p62 has been suggested as a shuttle protein to transport ubiquitinated substrates directly to the autophagosome (Pankiv et al., 2007). Recently, p62 has also been implicated in the autophagic clearance of non-ubiquitinatedSTAT5A_ΔE18 as well (Watanabe and Tanaka, 2011). In this study, knockdown of p62 inhibited LC3 lipidation and autophagosome formation, however accumulation of non-ubiquitinated STAT5A_ΔE18 was not observed suggesting incomplete inhibition of autophagy in p62 knockdown cells. Adapter proteins, such as Nbr1, have been shown to be recruited to ubiquitin aggregates where they are thought to play a role in autophagy similar to p62 to shuttle tagged proteins for autophagic clearance (Kirkin et al., 2009). Thus, even with knockdown of p62, some level of autophagy persists.

My data expands on the model where hyperactivated HDAC6 results in hypoacetylation of cortactin leading to a dramatic increase in recruitmentofcortactin to F-actin (Fig. 6A evs. Fig. 6B e).Interestingly, our data demonstrated that absence of p62 resulted in increased colocalization of cortactin-F-actin assemblies with aggregates containing HDAC6 during QC-autophagy (Fig. 3-6A vs. Fig. 3-6B merge for MG132-treated cells). However, perinuclear aggresome-like aggregates containing HDAC6 were seen to colocalize with cortactin/F-actin assemblies in WT cells while they accumulate in the cytoplasm of p62KO cells. It is possible that under basal conditions, the level of compensatory clearance pathways, such as Nbr1, did not show sufficient clearance of aggregates by our detection methods. However, when QC-autophagy was stimulated by protein misfolding, I would expect to see some clearance of aggregated proteins to the

MTOC even in the absence of p62. This was not the case as the localization of cortactin-F-actin assemblies remained localized to the cytoplasm in p62KO cells (Fig. 3-6B j).

Both HDAC6 and p62 are suggested to play independent roles in aggregate clearance (Yao, 2010). p62 is thought to be responsible for transporting aggregated proteins to the autophagosome (Pankiv et al., 2007) while HDAC6 is shown to be responsible for autophagosome-lysosome fusion (Lee et al., 2010a). Both proteins are implicated in transport of aggregated proteins to the aggresome for disposal (Yao, 2010). Based on our observation of direct interaction between the two proteins, I propose that p62 likely plays a dual role in autophagy, not only in the formation of the autophagosome, but also in the regulation of HDAC6 activity which is responsible for autophagosome-lysosome fusion. At basal levels, the ratio of p62 and HDAC6 maintain homeostasis of the autophagic process. However, absence of p62 leaves HDAC6 in a hyperactive state, resulting in excessive cortactin deacetylation and F-actin network assembly around aggregates (Fig. 3-6B e; Fig 3-6B k).

It is interesting to also postulate on the effects of hypoacetylation on α-tubulin. Acetylation of microtubules, comprised of both α and β-tubulin, is suggested to alter the structure and dynamics of microtubules (Tran et al., 2007, Zilberman et al., 2009). My data show that F-actin assemblies surrounding proteins aggregates remained in the cytoplasm in the absence of p62. While this could be due to a requirement for p62 for a direct attachment to motor proteins along the microtubule for retrograde transport to the MTOC, no such interaction between p62 and dynein has been shown. It is intriguing to suggest the deacetylase activity of HDAC6 affects the movement of motor proteins along

the microtubule. Under the control of p62 associating with HDAC6, tubulin acetylation could be regulated. However, if unregulated by the absence of p62, motor protein transport could be compromised which would prevent aggregate movement and aggresome formation. This is indeed the case for what is seen in the absence of p62 with cytoplasmic localization of cortactin-F-actin assemblies upon induction of protein misfolding. More investigation into the role of acetylation on microtubule dynamics is warranted to shed further light on this process.

Reversible protein acetylation is being recognized in a wide range of cellular processes as a regulatory mechanism. The functional significance of acetylation/deacetylation has in the past been relegated to our understanding of transcriptional control process in the nucleus of the cell (Sadoul et al., 2011). However, cytoplasmic acetylation/deacetylation of proteins is becoming more recognized as a regulatory event and HDAC6 is at the crux of cytoplasmic deacetylation. My results support a central role for p62 in the regulation of HDAC6 deacetylase activity.

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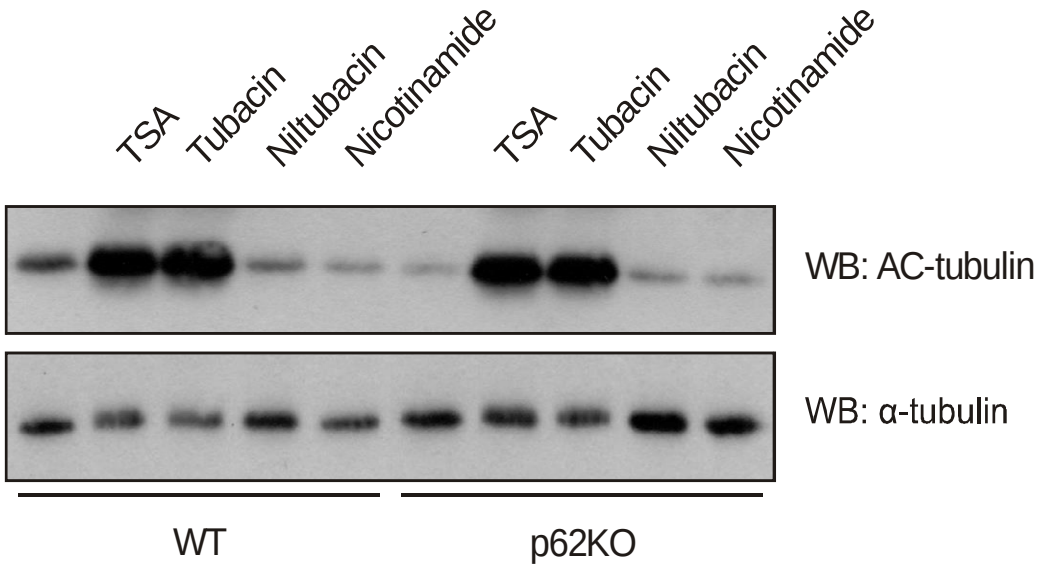
Figure 3- 1

A


TSA
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Nicotinamide
WB: AC-tubulin
WB: α-tubulin
WT
p62KO

B

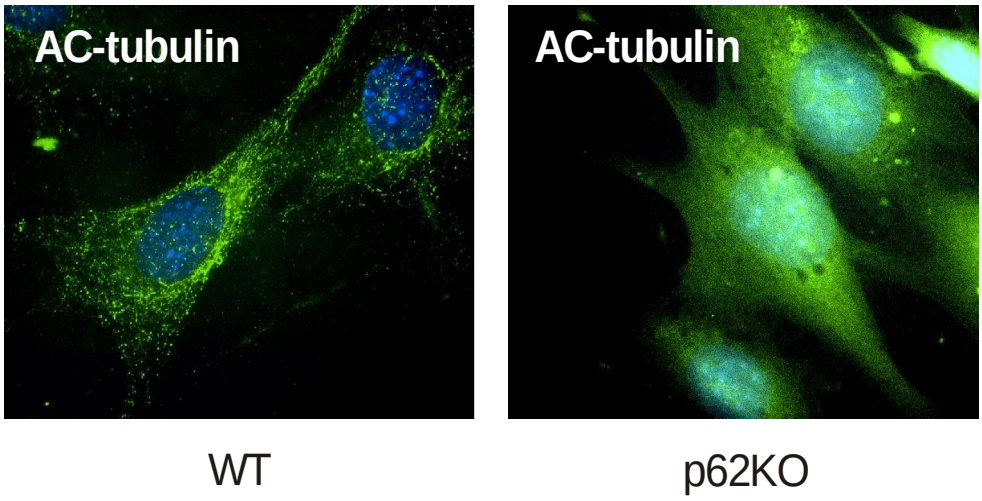
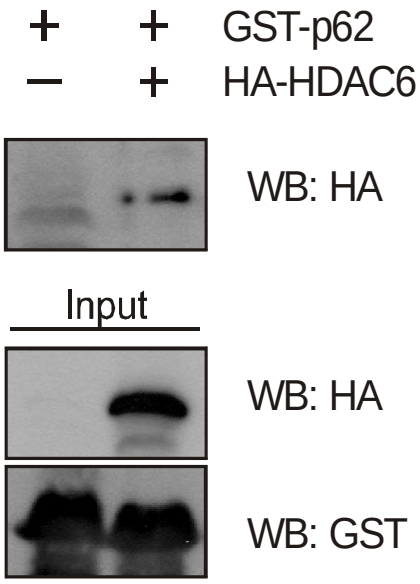

AC-tubulin
AC-tubulin
WT
p62KO

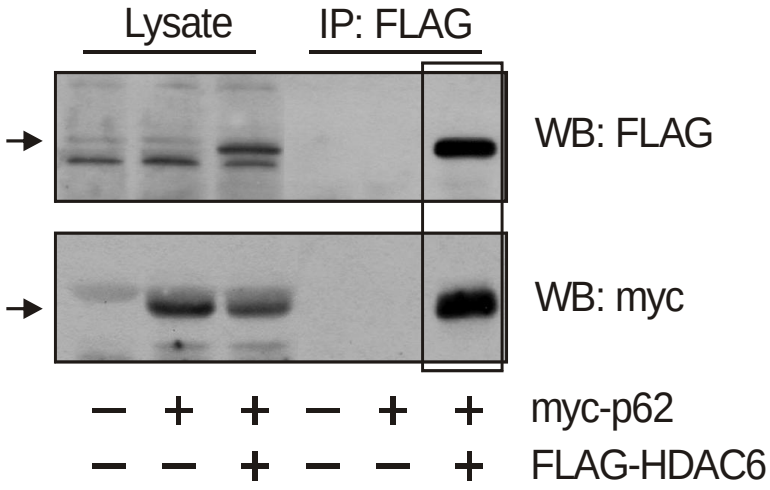
Figure 3-1.Absence of p62 leads to decreased acetylated-tubulin in mouse fibroblasts.(A) WT and p62KO MEF were treated with 2μM TSA, 10μM tubacin, 10μM niltubacin, or 10mM nicotinamide for 4 hours. Whole cell lysates were subjected to Western blot with anti-acetylated-α-tubulin and anti-α-tubulin antibodies. Blots were quantitated by densitometry and relative acetylated tubulin intensity was determined by normalizing the value of acetylated tubulin over α-tubulin. Analysis of the densitometry data for significance was performed using Student's *t*-tests. [WT vs. p62KO Untreated: $t(4)=29.03$, $p=4.19E-6$; WT vs. p62KO TSA: $t(4)=0.27$, $p=0.401$; WT vs. p62KO Tubacin: $t(4)=0.49$, $p=0.325$; WT vs. p62KO Nil-tubacin: $t(4)=0.71$, $p=0.259$; WT vs. p62KO Nicotinimide: $t(4)=0.32$, $p=0.382$] Data used for this analysis were from three independent replicates. (B) WT and p62KO MEF cells analyzed for acetylated α-tubulin by immunofluorescence.

Figure 3- 2

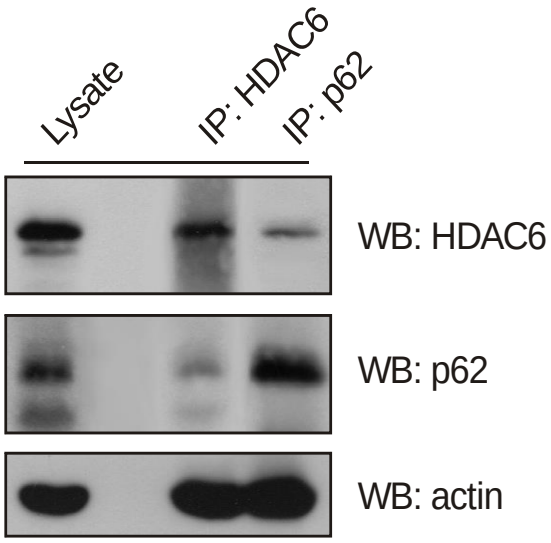
A


+ + GST-p62
– + HA-HDAC6
WB: HA
Input
WB: HA
WB: GST

B


Lysate
IP: FLAG
WB: FLAG
WB: myc
– + + – + + myc-p62
– – + – – + FLAG-HDAC6

C



D

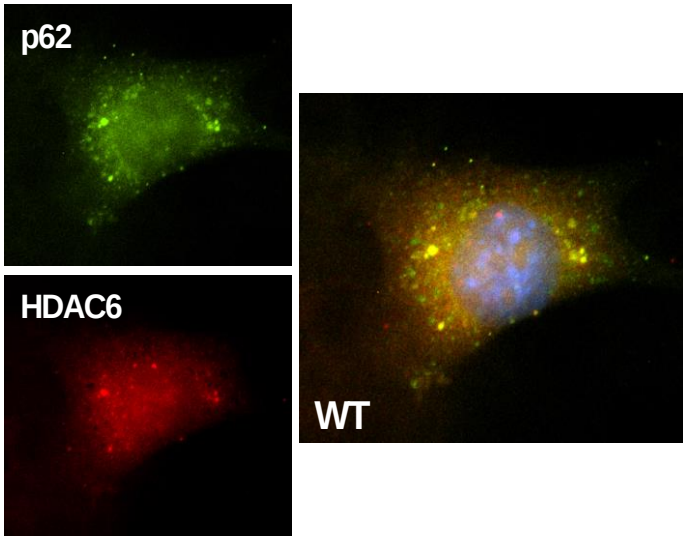
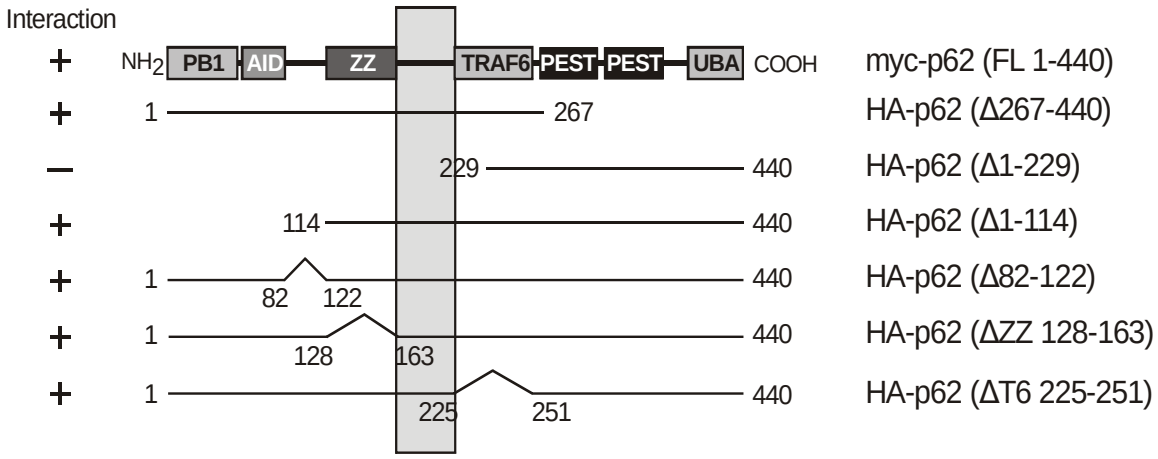


Figure 3-2.p62 specifically interacts with HDAC6. (A) Lysates of HEK cells transfected or not with HA-HDAC6 were subjected to GST-p62 pulldown assay. Tagged constructs were captured on glutathione sepharose beads and analyzed by Western blot with anti-HA and anti-GST antibodies. (B) HEK cells were transfected with FLAG-HDAC6 and myc-p62 constructs. HDAC6 was immunoprecipitated with FLAG-tag antibody and presence of co-precipitating myc-p62 (included in highlighted box) analyzed by Western blot with anti-myc antibody. Presence of transfected constructs were confirmed in the whole cell lysate. (C) Endogenously expressed p62 and HDAC6 were examined in WT MEF cells by immunoprecipitation with p62 or HDAC6 antibodies and analyzed by Western blot with corresponding antibodies. (D) Endogenous HDAC6 and p62 localization was examined by immunofluorescence in WT MEF cells.

Figure 3- 3

A


Interaction
NH2
PB1
AID
ZZ
TRAF6
PEST
PEST
UBA
COOH
myc-p62 (FL 1-440)
HA-p62 (Δ267-440)
HA-p62 (Δ1-229)
HA-p62 (Δ1-114)
HA-p62 (Δ82-122)
HA-p62 (ΔZZ 128-163)
HA-p62 (ΔT6 225-251)
1
267
229
440
114
82
122
128
163
225
251

B

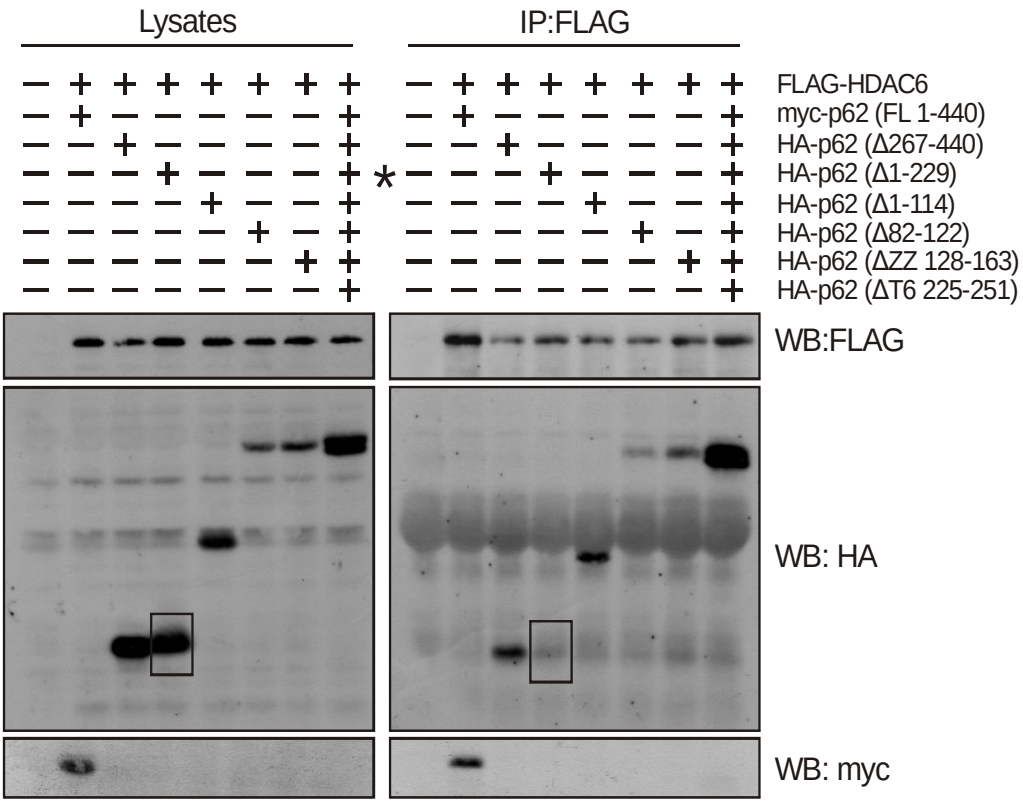
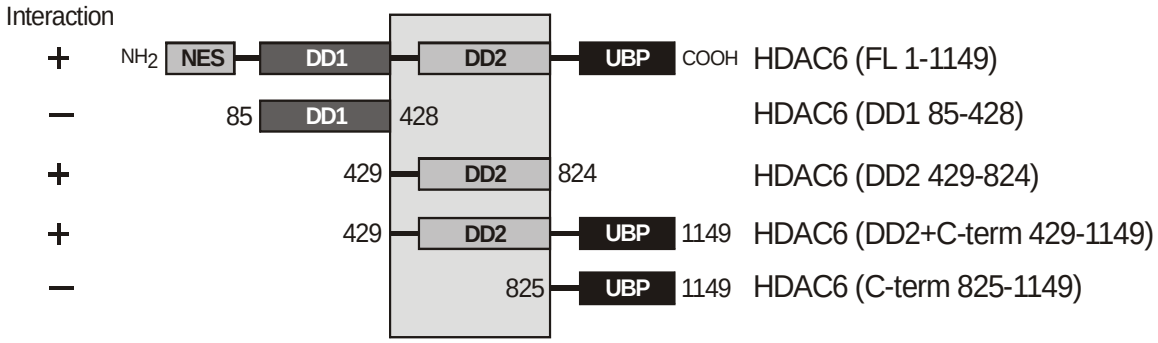

Lysates
IP:FLAG
FLAG-HDAC6
myc-p62 (FL 1-440)
HA-p62 (Δ267-440)
HA-p62 (Δ1-229)
HA-p62 (Δ1-114)
HA-p62 (Δ82-122)
HA-p62 (ΔZZ 128-163)
HA-p62 (ΔT6 225-251)
WB:FLAG
WB: HA
WB: myc

Figure 3-3.Mapping the interaction region on p62.(A) Schematic diagram of HA-tagged p62 deletion constructs: full length (FL) myc-p62 and HA-p62 with deletion (Δ) of aa 267-440, aa 1-229, aa 1-114, aa 82-122, ZZ domain (ZZ) and TRAF6 domain (T6) individually. (B) HEK cells were transfected with full length FLAG-HDAC6 and HA-tagged p62 deletion mutants constructs. Full length p62 was myc-tagged. HDAC6 was captured with anti-FLAG antibody and co-precipitating myc-tagged full length p62 or HA-tagged p62 deletion constructs analyzed by Western blot with anti-HA antibody. The rectangle boxed blots represent the only p62 deletion construct (HA-p62 Δ1-229) that was not immunoprecipitated with FLAG-HDAC6. The data presented was the representative blot from three independent replicates.

Figure 3- 4

A


Interaction
+
−
+
+
−
NH2
NES
DD1
DD2
UBP
COOH
HDAC6 (FL 1-1149)
85
DD1
428
HDAC6 (DD1 85-428)
429
DD2
824
HDAC6 (DD2 429-824)
429
DD2
UBP
1149
HDAC6 (DD2+C-term 429-1149)
825
UBP
1149
HDAC6 (C-term 825-1149)

B

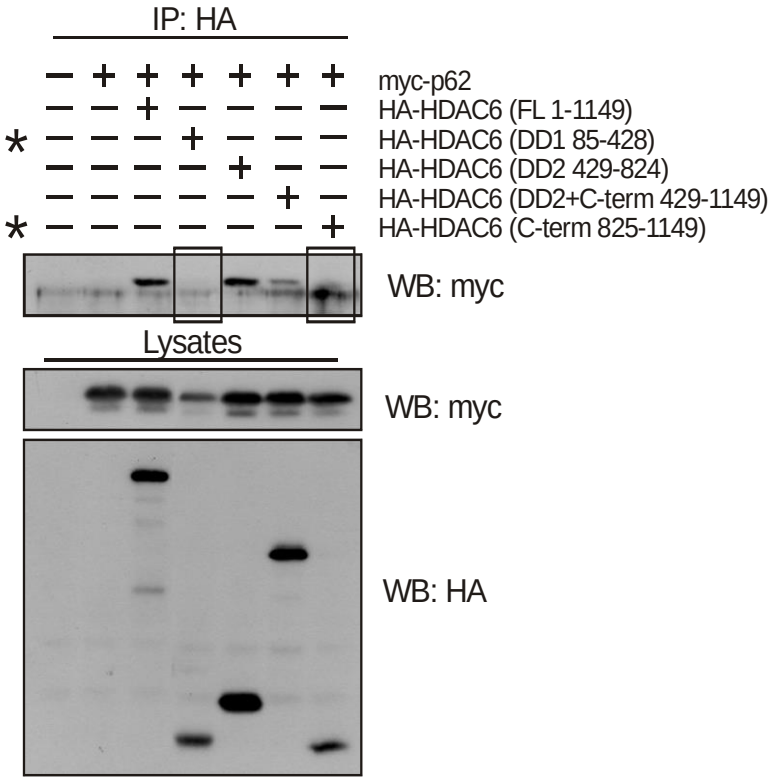

IP: HA
myc-p62
HA-HDAC6 (FL 1-1149)
HA-HDAC6 (DD1 85-428)
HA-HDAC6 (DD2 429-824)
HA-HDAC6 (DD2+C-term 429-1149)
HA-HDAC6 (C-term 825-1149)
*
*
WB: myc
Lysates
WB: myc
WB: HA

Figure 3-4.Mapping the interaction region on HDAC6.(A) Schematic diagram of HA-tagged HDAC6 truncation constructs. (B) HEK cells were transfected with full length myc-p62 and HA-tagged HDAC6 deletion constructs. p62 was captured with anti-myc antibody and co-precipitating HA-tagged HDAC6 constructs analyzed by Western blot with anti-HA antibody. The data presented was the representative blot from three independent replicates.

Figure 3- 5

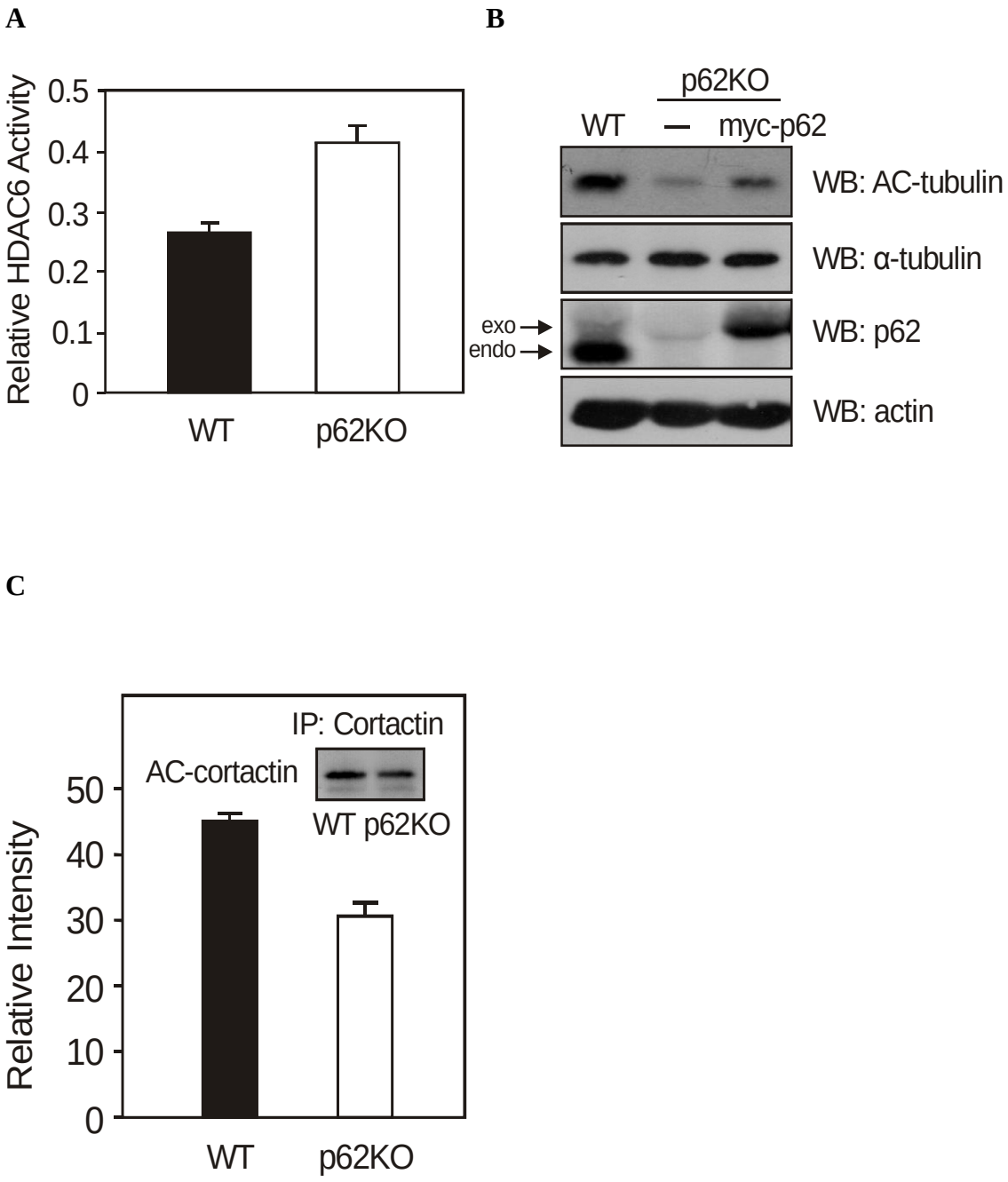
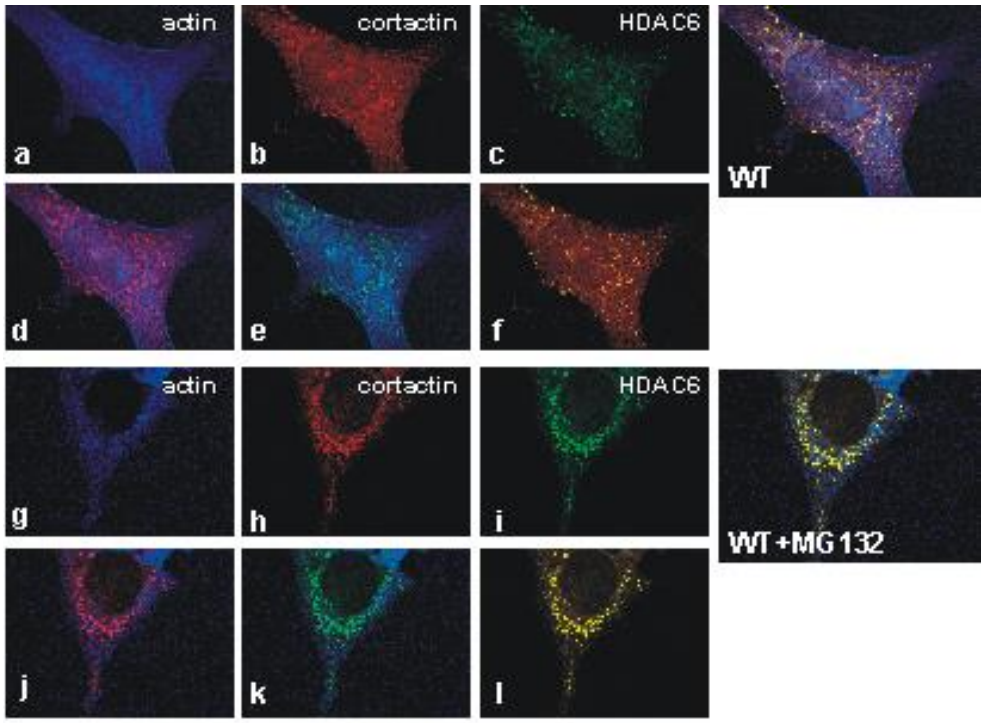

A
B
Relative HDAC6 Activity
0.5
0.4
0.3
0.2
0.1
0
WT
p62KO
p62KO
WT
myc-p62
exo
endo
WB: AC-tubulin
WB: α-tubulin
WB: p62
WB: actin
C
IP: Cortactin
AC-cortactin
WT p62KO
Relative Intensity
50
40
30
20
10
0
WT
p62KO

Figure 3-5. Absence of p62 enhances HDAC6 deacetylase activity.(A) Endogenous HDAC6 was immunoprecipitated with anti-HDAC6 antibody from WT and p62KO MEF cell lysates. HDAC6 immune complexes were incubated with acetylated lysine substrate. HDAC6specific activity was measured colorimetrically with a spectrophotometer. Relative HDAC6 deacetylation activity is presented from three separate experiments (one-tailed $t = 4.47$; $p < 0.01$). (B) myc-tagged full length p62 construct was transfected or not into p62KO MEF cells. Whole cell lysates were subjected to Western blot with anti-acetylated (AC)-α-tubulin, anti-α-tubulin, and anti-p62 antibodies. WT MEF cell lysates were loaded as control expression levels. Actin immuno-reactivity was included as a loading control and exogenous (exo) and endogenous (endo) bands of p62 are indicated. (C) Endogenous cortactin was immunoprecipitated from WT and p62KO MEF cells using anti-cortactin antibody and subjected to Western blot with anti-acetylated-cortactin. Relative acetylated cortactin intensity in WT and p62KO cells was calculated by normalizing acetylated cortactin over total cortactin on immunoprecipitates. Results are representative of three separate experiments (one-tailed $t = 6.80$; $p<0.05$).

Figure 3- 6

A

WT



B

p62KO

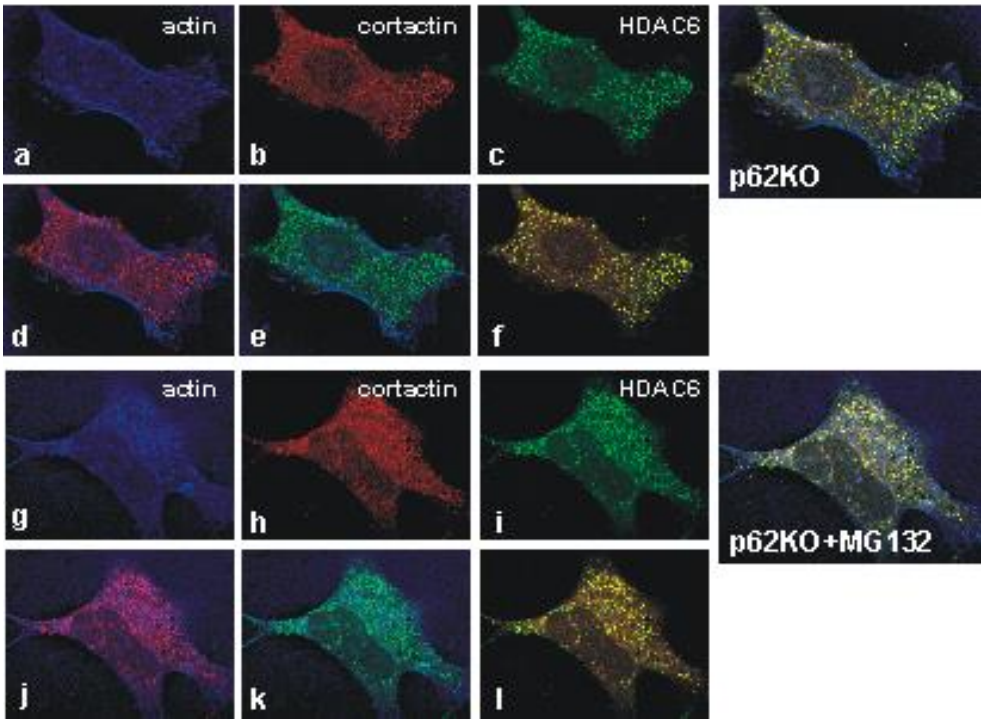


Figure 3-6.p62 regulates physiological function of cortactin-F-actin assembly. (A) WT MEF cells, either untreated (a-f) or treated (g-l), with 5µM MG132 for 6 hours were fixed with paraformaldehyde. F-actin was stained with phalloidin-AF350. Endogenous cortactin was immuno-stained with Texas Red secondary antibody and endogenous HDAC6 immuno-stained with Oregon Green secondary antibody. Cells were analyzed with confocal microscopy. Panel identification is as follows: a and g – actin; b and h – cortactin; c and i – HDAC6; d and j – actin/cortactin; e and k – actin/HDAC6; f and l – cortactin/HDAC6. A fully merged image is shown to the right of each set of panels. (B) p62KO MEF cells, either untreated (a-f) or treated (g-l), with 5µM MG132 for 6 hours, were subjected to the same immunofluorescence procedure as described above.

CHAPTER IV. SQSTM1/P62 REGULATES MICROTUBULE DYNAMICS

Abstract

Microtubules are dynamic elements of the cytoskeleton that participate in several cellular events. Microtubule dynamics can be regulated by a variety of microtubule-associated proteins (MAPs) through binding to different positions on microtubules. In addition, microtubule dynamics can be also regulated by post-translational modifications, such as acetylation, made to their tubulin subunits. The acetylation state of tubulin can be regulated by a balance between acetyltransferases and deacetylases. As a tubulin deacetylase, HDAC6 can regulate microtubule acetylation through deacetylation. In chapter 3, I provided evidence that the multi-functional signaling protein SQSTM1/p62 is a HDAC6-interacting protein and that absence of p62 in MEFs results in increased HDAC6 activity and decreased tubulin acetylation. P62 has been documented to interact with light chain 3 (LC3) of MAP1 and plays a critical role in aggresome formation which relies on microtubule-dependent transport. Due to the interaction between p62 and MAPs and its modulation effect on HDAC6 activity, I sought to examine whether microtubule dynamics are altered in p62 null background. Herein, I report that p62 does associate with microtubules in WT MEFs. Absence of p62 in MEFs stabilizes microtubules. Inhibition of HDAC6 by Trichostatin A (TSA) enhances its binding to microtubule which

had no effect on microtubule stability. These results indicate that absence of p62 rather than increased HDAC6 activity may contribute to altered microtubule stability in p62KO MEFs. Moreover, I observed increased microtubule reassembly in p62KO MEFs. This result supports a role of HDAC6 activity in regulation of microtubule growth.

Introduction

Microtubules are essential cytoskeleton components that participate in diverse cellular activities including cell division, cell migration, intracellular transport, and cell morphogenesis (Nagai et al., 2013). Microtubules are composed of cylindrical polymers of α-tubulins and β-tubulins. These two tubulin subunits associate in a head-to-tail manner to form heterodimers which link to generate linear protofilaments. Protofilaments come together and are sealed by lateral interaction to form the hollowed tube structure of microtubules. The polarized arrangement of tubulin heterodimers results in polarity of microtubules with exposure of α-tubulin at minus end near the MTOC and β-tubulin at plus end toward the cell periphery (Yvon and Wadsworth, 1997). Microtubules are highly dynamic structures that undergo frequent transition between polymerization anddepolymerization (Kawamura and Wasteneys, 2008). This is called dynamic instability which preferentially occurs at the plus end of microtubules (Galjart, 2005). Dynamic instability allows microtubule arrays to alter at a constant basis. This has been shown to be involved in several cellular functions of microtubules, such as rearrangement of microtubule networks during cell division and cell migration (Hage-Sleiman et al., 2011). It has also been associated with motor-mediated transport of proteins and

organelles along microtubules. Thus, regulation of microtubule dynamic instability is of central importance to microtubule-dependent cellular functions.

Microtubule dynamic instability could favor tubulin depolymerization which leads to turnover of microtubules. Because microtubules provide the track for trafficking of proteins or organelles to various cellular locations, maintaining the integrity of this track by preventing tubulin depolymerization is of great importance. Stabilization of microtubules can be achieved by binding of microtubule-associated proteins (MAPs) (Mandelkow and Mandelkow, 1995). MAPs include several types of proteins, such as the microtubule motor proteins dynein and kinesin and the plus-end-binding proteins CLIP-170 and EBs. These MAPs are generally considered to be enzymatically active (Dehmelt and Halpain, 2005). There is also a group of proteins including MAP1,2,4 and tau which bind along the lattice of microtubules. These MAPs are generally non-enzymatic and are named structural MAPs (Dehmelt and Halpain, 2005). The binding of structural MAPs to microtubules favors straight heterodimer conformation, thus holding protofilaments together. By doing this, most structural MAPs suppress the transition from polymerization to depolymerization (catastrophes) and stabilize microtubules by promoting their assembly. Interestingly, other MAPs, such as stathmin, through interaction with tubulin subunits, appear to destabilize microtubules by increasing frequency of catastrophes (Belmont and Mitchison, 1996). Thus, structural MAPs play an important role in regulation of microtubule dynamics to both directions. Binding of structural MAPs to microtubules is regulated by phosphorylation on these MAPs. Loss of phosphorylation generally leads to diminished binding of MAPs to microtubules and altered microtubule dynamics (Drewes et al., 1995, Di Paolo et al., 1997). Moreover,

genetic inactivation of MAPs leads to microtubule-dependent functional defects in the central nervous system (Liedtke et al., 2002, Rapoport et al., 2002).

It is also evident that post-translational modifications of microtubules are closely associated with microtubule dynamic properties (Hammond et al., 2008). These modifications include detyrosination, polyglutamylation, polyglycylation and acetylation. Of these modifications, acetylation is unique as it occurs on the luminal side of microtubules (Nogales et al., 1999). Thus, how microtubule acetylation is regulated has become an area of intense research interest. It has been found that tubulin is acetylated by *C. elegans* acetyltransferase MEC-17 and its mammalian homologue αTAT-1 and deacetylated by HDAC6 and SIRT-2 (Hubbert et al., 2002, North et al., 2003, Akella et al., 2010, Shida et al., 2010). Of these enzymes, HDAC6 was discovered the first and thus has been studied the most. HDAC6 is a unique member of class II histone deacetylases that is predominantly localized in cytoplasm (Verdel et al., 2000). HDAC6 directly interacts with α-, β-tubulin subunits as well as microtubules (Zhang et al., 2003). Acetylation was initially only found in stable microtubule species, thus it was considered to be a marker for stable microtubules(Piperno et al., 1987). Further research showed that acetylation also occurs on dynamic microtubule species and is associated with microtubule dynamic properties (Schatten et al., 1988, Chu and Klymkowsky, 1989, Lim et al., 1989). However, how acetylation affects microtubule dynamics remains unclear. Nevertheless, as a tubulin deacetylase, HDAC6 has been found to regulate microtubule dynamics. For instance, overexpression of HDAC6 has been shown to destabilize microtubules while genetic inactivation of HDAC6 increases microtubule stability

(Matsuyama et al., 2002, Tran et al., 2007). Moreover, HDAC6 activity is also involved in regulation of microtubule dynamics. Inhibition of HDAC6 activity by its specific inhibitor has been shown to delay microtubule regrowth or decrease microtubule assembly rate in cells (Zilberman et al., 2009, Asthana et al., 2013).

In our laboratory's recent study, I identified the multifunctional protein p62 as an HDAC6 interacting protein and lack of p62 in MEFs led to increased HDAC6 activity and reduced tubulin acetylation. P62 is connected to several signaling pathways through its multiple protein interaction domains. In particular, p62 has a LIR domain that interacts with Light chain 3 of MAP1 which is an autophagosome protein in autophagy pathway (Pankiv et al., 2007). Moreover, p62 plays an important role in protein aggregation pathway through recruitment of ubiquitinated proteins by its UBA domain and oligomerization by its PB1 domain (Bjorkoy et al., 2005). Aggregates accumulated by p62 are believed to collect at the MTOC through retrograde transport on microtubules to form aggresomes. Therefore, it is possible that the presence of p62 may be associated with microtubule behavior, such as dynamics. Based on this information, I sought to determine whether p62 is associated with microtubules and whether presence of p62 plays a role in regulation of microtubule dynamics. I therefore studied stability and microtubule reassembly rate in WT and p62KO MEFs. My results indicate that lack of p62 in MEFs did stabilize microtubules. However, while inhibition of HDAC6 by TSA in p62-deficient MEFs enhanced its binding to microtubules, it had no effect on microtubule stability. Finally, I show that the rate of microtubule reassembly increased in p62-

deficient MEFs. My results overall indicate that p62 may play a significant role in maintaining microtubule dynamics.

Materials and Methods

Cell Culture

Wild Type (WT) and p62 knock-out (p62KO) mouse embryonic fibroblasts (MEF) cells were grown in DMEM supplemented with 10% Fetal Calf Serum and antibiotics at 37^{o}C with high humidity and 5% CO_2.

Antibodies and Reagents

Monoclonal HDAC6 antibody was obtained from Cell Signaling (Danvers, MA). Monoclonal anti-p62 and polyclonal anti-α-tubulin antibodies were purchased from Abcam (Cambridge, MA). Monoclonal anti-α-tubulin antibody, anti-acetylated-α-tubulin antibody,taxol, nocodazole and trichostatin A (TSA) were obtained from Sigma (St. Louis, MO).

Western Blot

Either MEF or HEK cells were lysed on ice using Triton Lysis Buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 10mM NaF, 0.5% Triton X-100, 1mM Na_3VO_4, 1mM PMSF, 2µg/ml aprotinin and leupeptin). Protein concentration was determined by Bradford Assay (Bio-Rad, Hercules, CA). Samples were separated by SDS-PAGE followed by transfered to nitrocellulose membrane.The membrane was then blocked by 7% milk in TBS/Tween followed by incubation with primary and secondary antibodies.

Microtubule Fractionation

WT and p62KO MEFs were trypsinized and pelleted. Pellets were then washed and resuspended in PME buffer (0.1M PIPES, 1mM $MgCl_2$, 2mM EGTA, 2mM dithothreitol supplemented with 0.1M GTP, 1mM PMSF, 2μg/ml aprotinin, and 2μg/ml leupeptin). Cells were lysed by sonication and homogenization on ice. Microtubules in lysates were depolymerized by incubation on ice for 15 minutes. Lysates were then centrifuged at 10,000X g at 4℃ for 15 minutes. Supernatants containing microtubules were transferred to microfuge tubes followed by addition of GTP (final concentration 1mM) and taxol (final concentration 20μM). Microtubules were pelleted from supernatants after incubation at 37℃ for 30 minutes. Microtubule pellets were resuspended in PME buffer. Microtubule fractions, second time supernatant, along with whole cell lysate were quantitated and subjected to Western blot.

Microtubule Regrowth Assay

WT and p62KO MEFs were treated with nocodazole at 10μg/ml for 3 hours. Cells were then washed with PBS and fresh DMEM followed by growing in nocodazole-free media for 5 and 10 minutes. To fractionate polymerized and unpolymerized tubulin, cells were incubated with MTB buffer (20mM Tris-HCl pH 6.8, 1mM $MgCl_2$, 2mM EGTA, 0.5% NP-40, 0.14M NaCl) supplemented with 4.7μM taxol and protease inhibitors at 37°C for 10 minutes. Cells were scraped off from the plate and transferred to microfuge tube followed by centrifuge at 12,000g for 15 minutes at 4°C. The supernatant was then removed and saved as the unpolymerized tubulin fraction. The pellet was resuspended in

H2O as polymerized tubulin fraction. Samples from both fractions were subjected to Western Blot as described above.

Microtubule Stability Assay

WT and p62KO MEFs were treated with nocodazole at different concentrations. Polymerized and unpolymerized tubulin were fractionated as above. Samples of both fractions for each treatment were subjected to Western blot.

Immunofluorescence Microscopy

Cells were fixed in 4% paraformaldehyde in PBS for 1 hour at 37°C. Cells were then pemeablized in 0.1% Triton X-100 in PBS for 10 minutes followed by blocking in 3% milk/PBS for 4 hours. P62 and α-tubulin were probed by incubation with mouse monoclonal anti-p62/rabbit polyclonal anti-α-tubulin antibodies (Abcam) or mouse monoclonal anti-α-tubuin mouse antibody (Sigma) alone at 4°C overnight. The protein-antibody complex was further probed with fluorescently tagged anti-mouse Oregon green/anti-rabbit Texas red or anti-mouse Oregon green secondary antibodies (Life Technologies, Grand Island, NY) at room temperature for 2 hours. The coverslips were mounted to slides. Colocalization of p62 with microtubules or microtubule network was examined by fluorescence microscopy.

Results

P62 is associated with microtubules

p62 interacts with microtubule-associated protein HDAC6 and tau (Yan et al., 2013, Babu et al., 2005). To investigate the possible effect of p62 on microtubule dynamics, I first wanted to examine if p62 preferentially localizes to microtubules. Whole cell lysates were obtained from WT MEFs followed by microtubule fractionation. P62KO MEFs were included as a control. I observed a significant increase of p62 in microtubule-enriched fraction (pellet) from WT MEFs compared to that in whole cell lysate (Fig. 4-1A). Interestingly, there was almost no p62 detected in the supernatant fraction which contains both cytoplasmic and nuclear proteins (Fig.4-1A). As p62 is a cytoplasmic protein, this result was probably due to insufficient exposure time of Western Blot. However, noticeable amount of p62 was detected in the pellet under the same exposure time, indicating p62 is largely resided in the area of microtubules rather than other regions in the cytoplasm. To further confirm localization of p62 on microtubules, colocalization of endogenous p62 and microtubules in WT MEFs was examined by immunostaining with anti-p62 and anti-α-tubulin antibodies. Substantial amount of p62 was observed to colocalize with microtubule networks (Fig. 4-1B). Collectively, these results suggest that p62 is associated with microtubules in WT MEFs.

Absence of p62KO in MEFs leads to increased microtubule stability

Association of p62 with microtubules implies that p62 may either directly interact with microtubule subunit tubulin or indirectly associate with microtubules through an

adaptor protein, such as HDAC6. Either type of interaction may have impact on microtubule dynamics, such as stability. Therefore, I next wanted to examine if loss of this interaction in p62KO MEFs alters microtubule stability. Microtubule stability can be assessed by examining the resistance of microtubules to microtubule depolymerizer. Therefore, to investigate whether microtubule stability is changed in p62 KO MEFs, both WT and p62KO MEFs were treated with 100nM and 300nM of the microtubule depolymerizer nocodazole respectively. To determine the resistance of microtubules upon treatment of nocodazole, polymerized and unpolymerized tubulin fractionation was performed on these cells. At each treatment of nocodazole (100nM and 300nM), more polymerized alpha-tubulin was observed in p62KO MEF fractions compared to WT MEF fractions (Fig. 4-2A). Therefore, the trend for decrease of polymerized tubulin in p62KO MEFs upon treatment with increased concentrations of nocodazole was slower than that in WT MEFs (Fig. 4-2B). To confirm the results from this biochemical approach, the amount of polymerized tubulin in WT and p62KO MEFs upon depolymerization by nocodazole was also assessed by immunofluorescence microscopy. At 100nM nocodazole treatment, while substantial amounts of polymerized microtubules were observed in p62KO MEFs, it was evident that some microtubules networks at the periphery of WT MEFs was depolymerized (Fig. 4-2C). At 300nM of nocodazole treatment, an intact microtubules network was still observed in p62KO MEFs while most microtubules were depolymerized in WT MEFs (Fig. 4-2C). Collectively, these results show that microtubules become more resistant to the microtubule depolymerizer nocodazole in p62KO MEFs, thus indicating increased microtubule stability in MEFs lacking p62.

TSA treatment enhances HDAC6 binding to microtubules in p62KO MEFs

A recent study has shown that pharmacological inhibition of HDAC6 enhances binding of HDAC6 to microtubules and increases microtubule stability in MCF-7 cells (Asthana et al., 2013). We therefore wanted to examine whether HDAC6 inhibition also alters its binding to microtubules and microtubule stability. p62KO MEFs were treated with broad class II HDAC inhibitor TSA. The treated p62KO MEFs and non-treated p62KO MEFs were destabilized by 10µg/ml of nocodazole for different periods of time and subjected to polymerized tubulin fractionation and Western blot. Interestingly, a increase in HDAC6 was observed in polymerized tubulin fractions from TSA treated p62KO MEFs compared to non-treated p62KO MEFs (Fig. 4-3A, B), indicating enhanced binding of HDAC6 to microtubules upon inhibition. However, a comparable level of polymerized tubulin was observed at each treatment time in p62KO MEFs treated with TSA, indicating that inhibition of HDAC6 did not increase microtubule stability.

Increased microtubule regrowth in p62KO MEFs

Microtubules are dynamic cellular structures that undergo constant polymerization and depolymerization. Therefore, the polymerization rate of microtubules is another important microtubule dynamic property. Pharmacological inhibition of HDAC6 has been shown to decrease microtubule growth rate or delay microtubule regrowth in cells (Zilberman et al., 2009, Asthana et al., 2013). In my experiments, I observed increased HDAC6 activity in p62KO MEFs. Therefore, I asked if microtubule growth is affected in p62KO MEFs. Microtubule regrowth assays were performed on WT

and p62KO MEFs. At both 5 minutes and 10 minutes regrowth time, much more α-tubulin was observed in polymerized tubulin fractions from p62 KO MEF compared to those in WT MEF(Fig. 4-4A and 4-4B). To further confirm altered microtubule regrowth in p62KO MEFs, these cells were subjected to the same microtubule regrowth protocol followed by immunofluorescence microscopy. As expected, while a substantial amount of α-tubulin became repolymerized upon regrowth over 5 and 10 minutes in p62KO MEFs, most α-tubulin still remained in an unpolymerized state in WT MEFs (Fig. 4-4C). Collectively, these results indicate that microtubules from p62KO MEFs possess different dynamic properties with increased regrowth rate.

Discussion

Microtubule dynamics can be regulated by a variety of microtubule proteins. These include MAP1, MAP2, tau, and HDAC6 that bind to the lattice of microtubules (Hubbert et al., 2002, Dehmelt and Halpain, 2005). Alternatively, plus-end binding proteins, such as TIPs, can also modulate microtubule dynamics through specific binding to the plus end (Akhmanova and Steinmetz, 2010). In addition, members of the microtubule motor protein family dynein and kinesin, such as kinesin-2, have also been shown to impact microtubule dynamics (Laan et al., 2012, Gumy et al., 2013). The activity of these microtubule proteins is controlled by signaling pathways that incorporate upstream regulators, such as kinases, in several different manners. For instance, GSK3β phosphorylation has been reported to regulate microtubule dynamics (Goold et al., 1999, Scales et al., 2009). Phosphorylation of tau by GSK3β suppresses its microtubule-stabilizing function (Wagner et al., 1996). Moreover, enzymes that control the acetylation,

polyglutamylation, detyrosination, and poly-glycylation state of microtubules can also regulate microtubule dynamics by altering binding of motor proteins to microtubules (Dompierre et al., 2007, Ikegami et al., 2007, Dunn et al., 2008, Verhey and Hammond, 2009). In this study, I clearly demonstrated that microtubule stability and reassembly rate are altered in MEFs with the absence of p62 compared to that in WT MEFs. These results indicate that p62 may play an essential role in maintaining dynamic properties of microtubules. Future study would be needed to identify the signaling pathway in which p62 is involved to regulate microtubule dynamics.

In the current study, I observed increased microtubule stability in p62KO MEFs. In Chapter 3, I report that reduced tubulin acetylation and increased HDAC6 activity occurs in p62KO MEFs. Interestingly, it has been documented that TSA-induced tubulin acetylation stabilizes microtubules (Matsuyama et al., 2002). If this is also the case in my cell model, I would have expected to observe decreased microtubule stability in p62KO MEFs. The contradictory data can be explained by the possibility that altered tubulin acetylation may not be the sole factor that contributes to microtubule stability in p62KO MEFs. In fact, microtubule stability can be also regulated by microtubule-associated proteins. Interestingly, p62 has been shown to interact with tau, a MAP that stabilizes microtubules through binding to lattice (Takemura et al., 1992, Al-Bassam et al., 2002). Thus, it would be tempting to examine whether the p62-tau interaction could affect tau's stabilizing effect on microtubules. Alternatively, it is also possible that p62 may affect function of other microtubule-associated proteins through interaction. In addition, I showed that p62 was enriched in microtubule fractions and that it colocalized with microtubule networks in WT MEFs. Thus, it is also interesting to postulate that p62 may

destabilize microtubules by functioning as a MAP itself through direct interaction with tubulin.

TSA is a broad class II HDAC inhibitor that can inhibit HDAC6 tubulin deacetylase activity. Therefore, my finding of increased HDAC6 binding to microtubules upon TSA treatment in p62KO MEFs supports the results from MCF-7 cells. Interestingly, TSA treatment did not increase microtubule stability in p62KO MEFs. While this discrepancy may be due to different experimental protocols or different cell types in these two studies, I do clearly show that hyperacetylation of tubulin induced by TSA treatment in p62KO MEFs did not stabilize microtubules. This supports previous findings that tubulin acetylation does not affect microtubule stability (Haggarty et al., 2003, Palazzo et al., 2003). My finding thus once again raises the question whether tubulin acetylation plays a role in microtubule stabilization or is just a consequence of microtubule stabilization.

In investigating another dynamic property of microtubules, I examined microtubule reassembly rates in both WT and p62KO MEFs. Both biochemical and immunofluorescence approaches showed that lack of p62 in MEFs leads to an increased microtubule reassembly rate, thus suggesting a different microtubule growth phenotype in these cells. As HDAC6 activity is elevated in p62KO MEFs, elevation of microtubule regrowth rate in p62KO MEFs may further support an essential role of HDAC6 activity in microtubule growth. In addition, I for the first time show that hyperactivation of HDAC6 may further increase microtubule regrowth rate upon the WT level. While increased HDAC6 activity is likely to contribute to altered microtubule regrowth rate in

p62KO MEFs, the possible involvement of the other factors subject to modification in p62KO MEFs cannot be excluded. Therefore, the future research should examine multiple factors involved in this regulatory process in p62KO MEFs.

Dynamic instability refers to the exchange rate associated with the polymerization and depolymerization cycle of microtubules. Thus, dynamic instability is an intrinsic property of microtubule dynamics. Stabilization of microtubules often suppresses dynamic instability by decreasing the rate of tubulin depolymerization. Thus, stabilization of microtubules in p62KO MEFs may inhibit their dynamic instability. Coincidentally, microtubule dynamic instability has been shown to be essential for aggresome formation in oligodendroglial cells upon proteolytic stress (Bauer and Richter-Landsberg, 2006). This finding indicates that trafficking of proteins to aggresomes is not only dependent on an intact microtubule railway track but also requires dynamic networks of microtubules (Bauer and Richter-Landsberg, 2006). My finding that microtubule stability increases in p62KO MEFs suggests a suppressed microtubule dynamic instability in these cells. Therefore, this possible suppression of microtubule dynamic instability may shed light on aggresome-deficiency phenotype of cells with depletion of p62. It can be postulated that this inhibited dynamic instability may lead to the trafficking defect and consequent abrogated aggresome formation in p62KO MEFs. Future study is needed to evaluate and complete this model.

In conclusion, in this portion of my research I show that microtubules from p62KO MEFs have different dynamic properties with increased stability and microtubule regrowth rate. My results support a role for HDAC6 activity in the regulation of

microtubule regrowth rate. In addition, increased microtubule stability and possible suppressed dynamic instability in p62KO MEFs indicates that p62 may affect aggresome formation by impacting microtubule trafficking.

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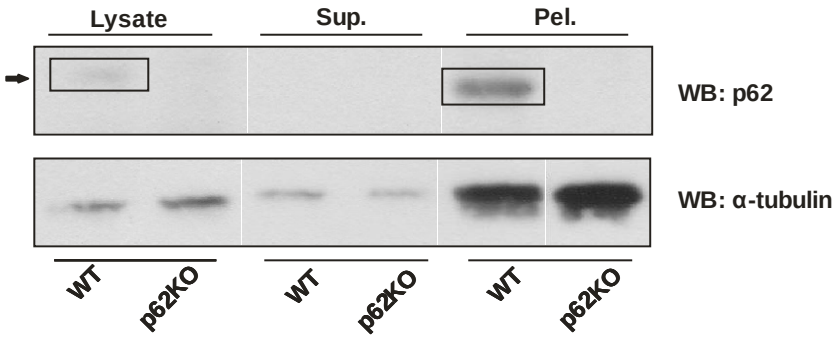
Figure 4- 1

A


Lysate
Sup.
Pel.
WB: p62
WB: α-tubulin
WT
p62KO
WT
p62KO
WT
p62KO

B

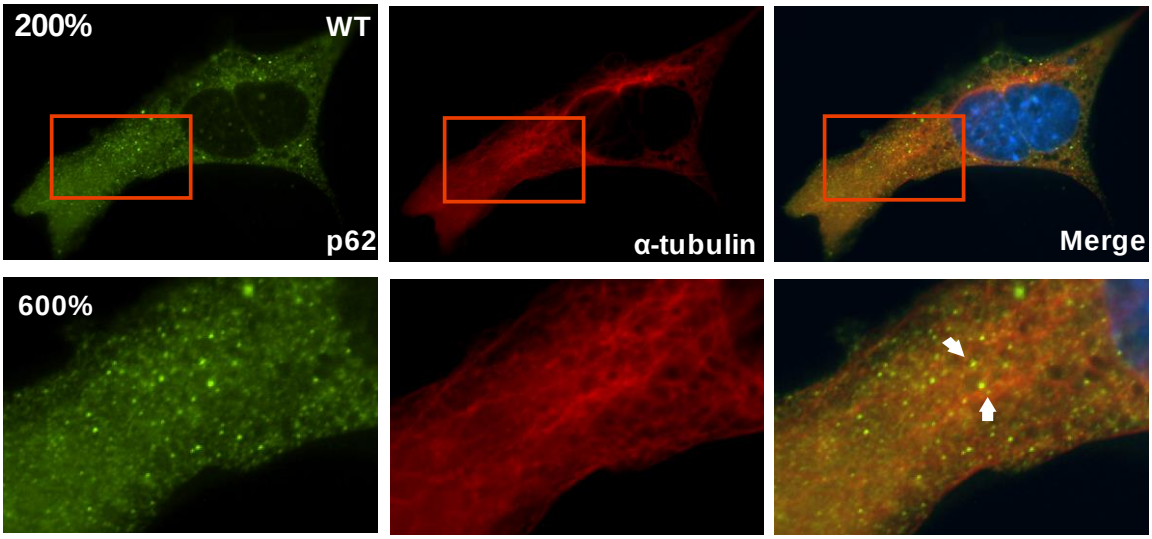
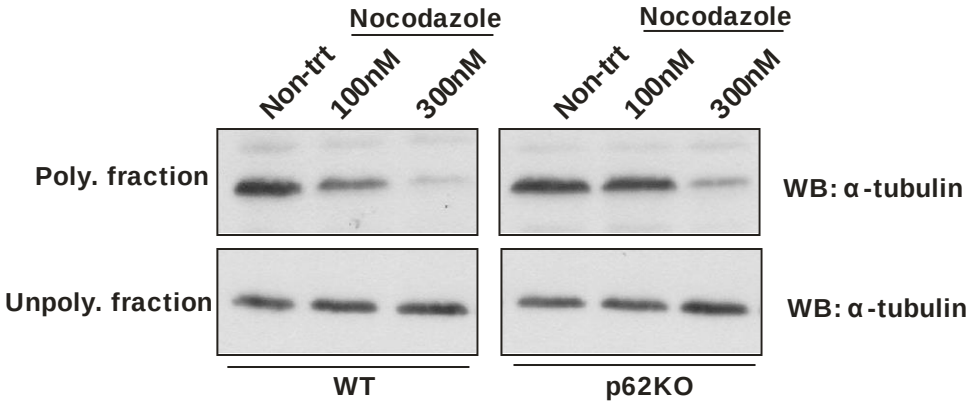

200%
WT
p62
α-tubulin
Merge
600%

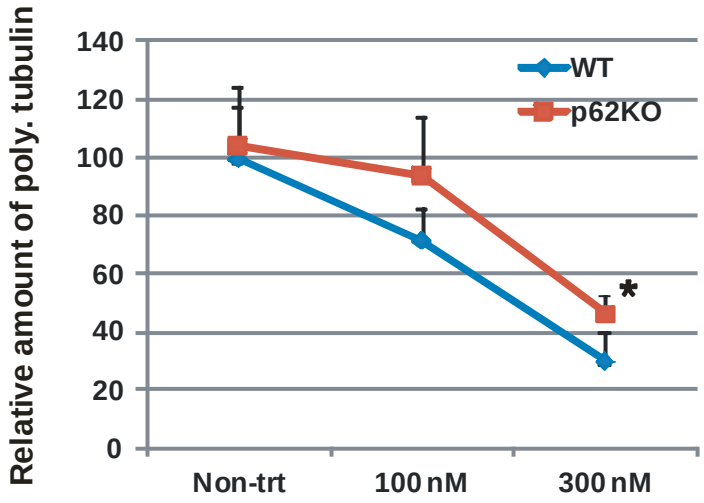
Figure 4-1. Association of p62 with microtubules. (A) Enriched microtubule fractions were pelleted from whole cell lysates of WT and p62KO MEFs. Pellets along with supernantants and whole cell lysates were subjected to Western blot with anti-p62 and anti-α-tubulin antibodies. The data is the representative blot from three independent replicates. (B) WT MEFs were fixed and immunostained with anti-p62 and anti-α-tubulin antibodies. The cocalization of p62 and α-tubulin was examined by fluorescence microscope.

Figure 4- 2

A


Nocodazole
Nocodazole
Non-trt
100nM
300nM
Non-trt
100nM
300nM
Poly. fraction
Unpoly. fraction
WB: α-tubulin
WB: α-tubulin
WT
p62KO

B


Relative amount of poly. tubulin
140
120
100
80
60
40
20
0
WT
p62KO
*
Non-trt
100 nM
300 nM

C

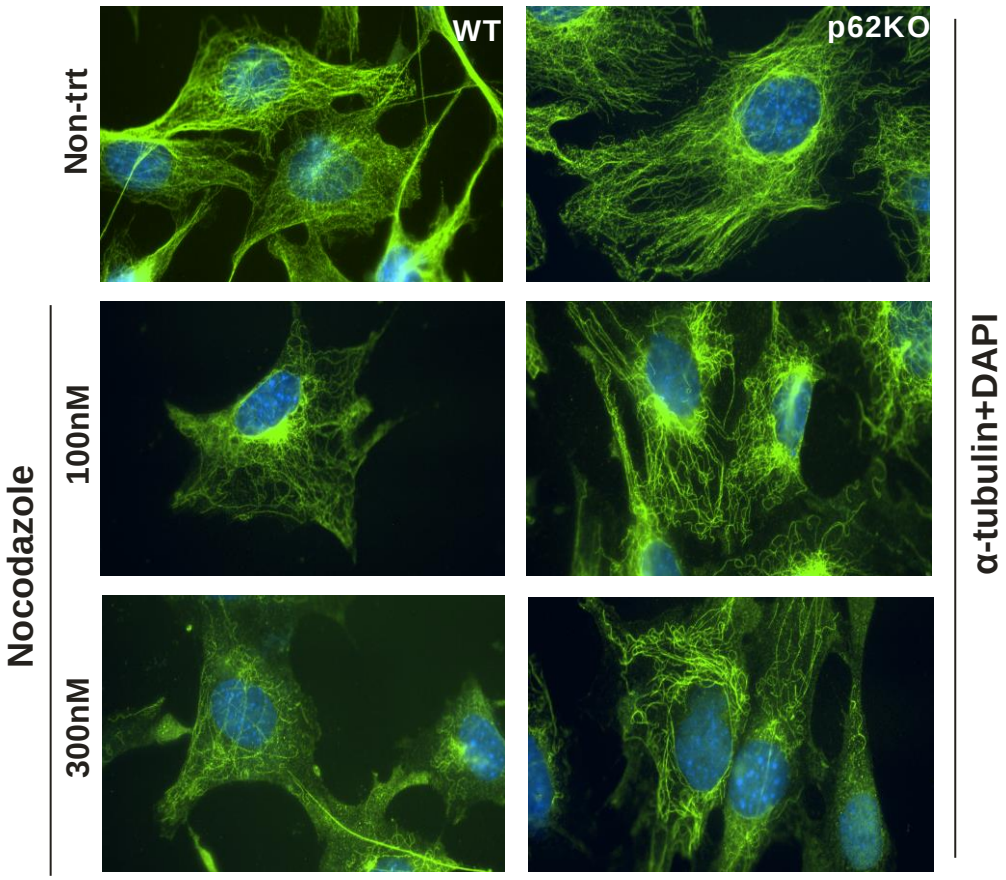
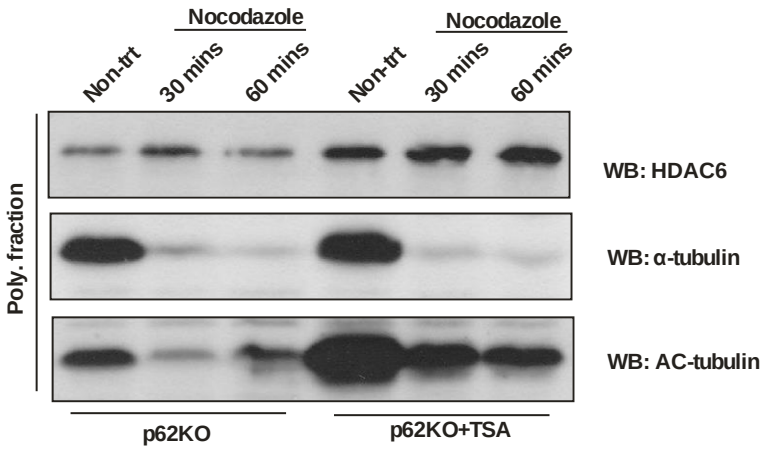


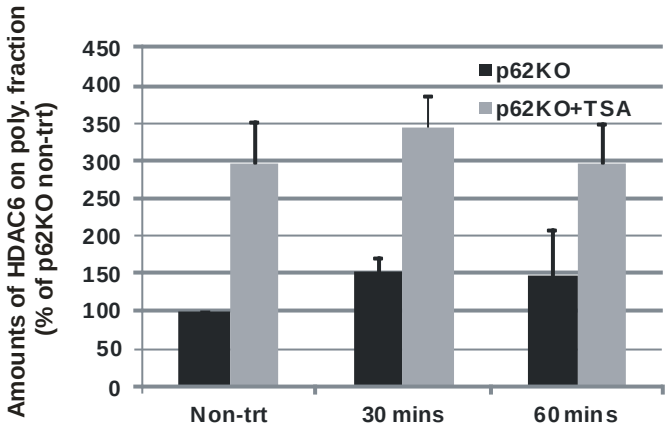
Figure 4-2. Absence of p62 in MEFs stabilizes microtubules. (A) Microtubules were depolymerized by 100nM or 300nM nocodazole in both WT and p62KO MEFs. Polymerized and unpolymerized tubulin fractionation was performed followed by Western blot with anti-α-tubulin antibody. (B) The relative amounts of polymerized tubulin were calculated using the densitormetry data from Western blot. The following formula was followed for calculation: relative polymerized tubulin = polymerized tubulin / polymerized tubulin + unpolymerized tubulin at each time point. The rate of microtubule depolymerization between WT and p62KO MEFs was calculated by using a linear regression model and compared by paired T test ($p=0.0385$). * represents the slope of linear regression from p62KO MEFs is significantly different ($\alpha=0.05$) from that of WT MEFs. Data presented was the average of three independent replicates. (C) WT and p62KO MEFs were treated with same concentrations of nocodazole in (A) followed by immunostaing with anti-α-tubulin antibody. The amounts of polymerized tubulin upon nocodazole treatment were examined by fluorescence microscope.

Figure 4- 3

A



B



C

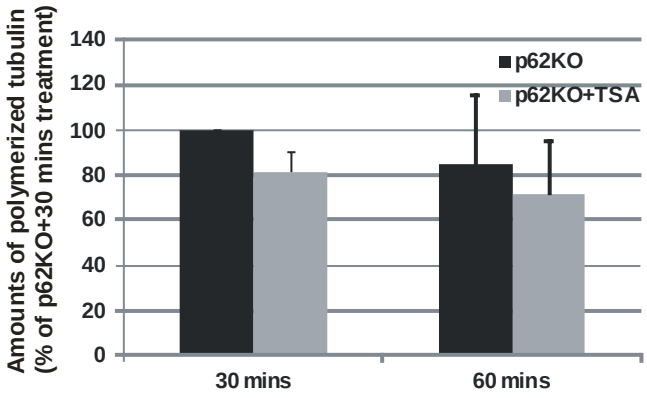
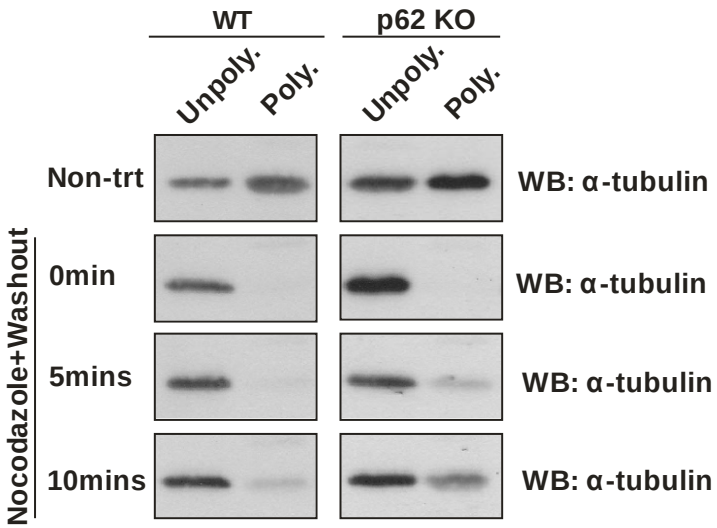


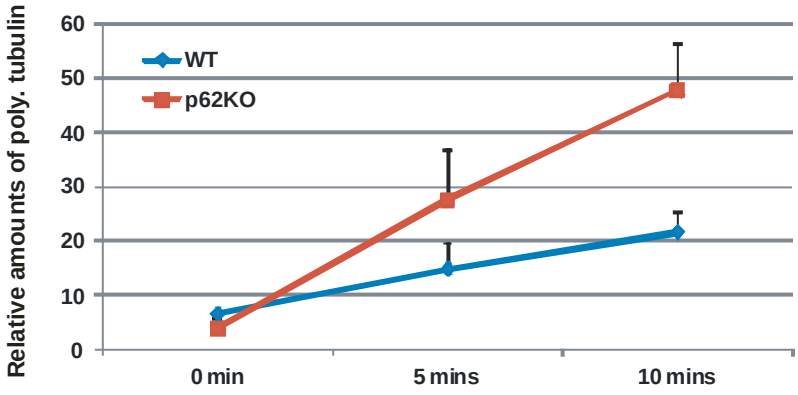
Figure 4-3. Effect of TSA treatment on HDAC6 binding and microtubule stability in p62KO MEFs. (A) p62KO MEFs were treated with 500nM TSA followed by nocodazole treatment at 10µg/ml for 30minutes and 60 minutes. Polymerized tubulin was fractionated from both treated and non-treated p62KO MEFs. These fractions were then subjected to Western blot with anti-HDAC6, anti-α-tubulin, and anti-acetylated-α-tubulin antibodies. (B) To compare amounts of HDAC6 associated with polymerized tubulin fraction at each treatment between p62KO and p62KO+TSA MEFs, the densitometry data of HDAC6 blot in (A) was graphed. (C) To compare amounts of polymerized tubulin between p62KO and p62KO+TSA MEFs, the densitometry data of α-tubulin blot in (A) was graphed.

Figure 4- 4

A


WT
p62 KO
Unpoly.
Poly.
Unpoly.
Poly.
Non-trt
WB: α-tubulin
Nocodazole+Washout
0min
WB: α-tubulin
5mins
WB: α-tubulin
10mins
WB: α-tubulin

B


Relative amounts of poly. tubulin
60
50
40
30
20
10
0
WT
p62KO
0 min
5 mins
10 mins

C

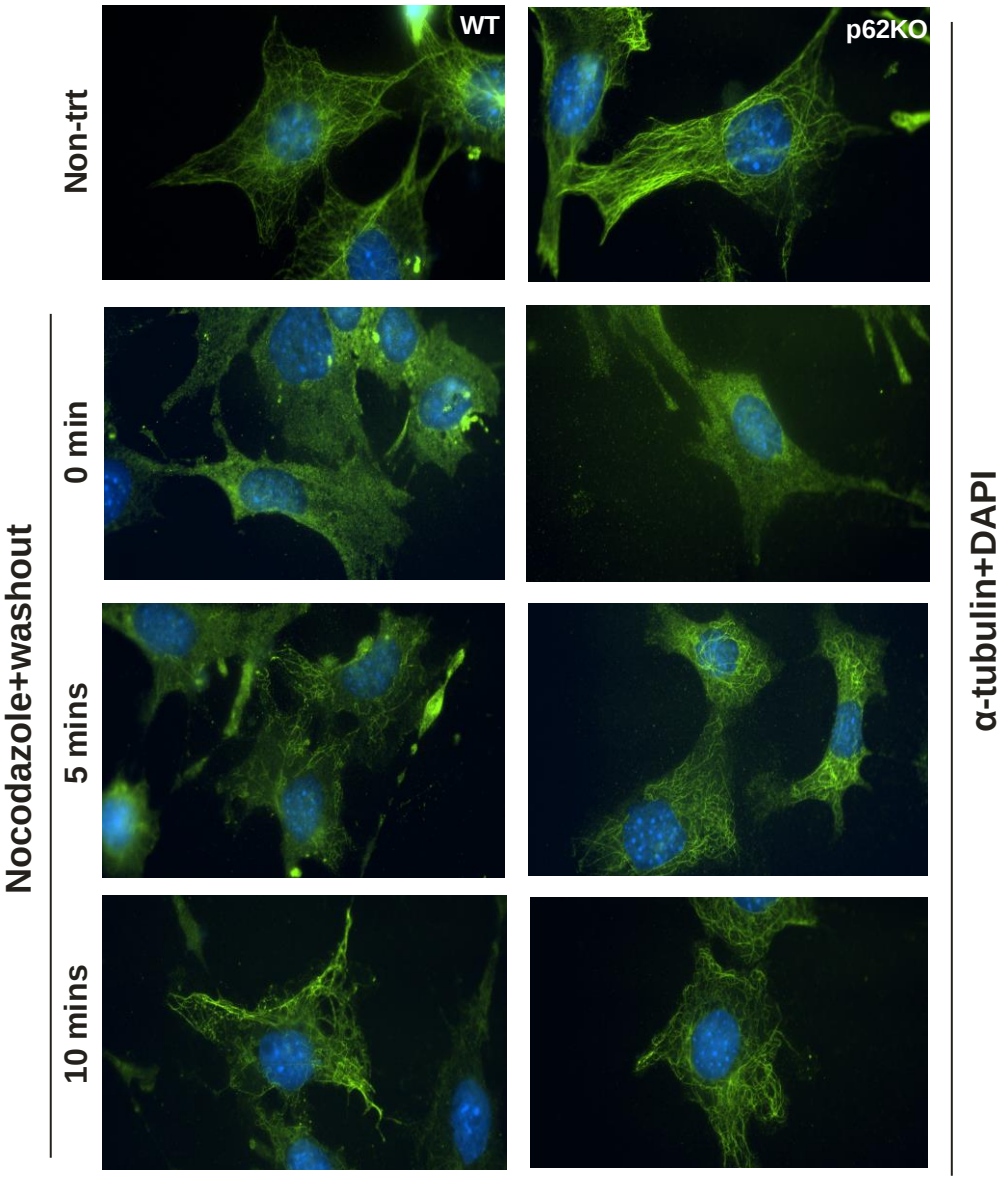

WT
p62KO
Non-trt
Nocodazole+washout
0 min
5 mins
10 mins
α-tubulin+DAPI

Figure 4-4. Increased microtubule regrowth rate in p62KO MEFs. (A) Microtubules in WT and p62KO MEFs were depolymerized by nocodazole followed by washing and regrow in nocodazole-free media for 5 minutes and 10 minutes. These cells were then subjected to polymerized and unpolymerized fractionation and Western blot. (B) The relative amounts of polymerized tubulin were calculated as Figure 4-2 (B). The data represented is the average of three independent experiments. (C) Microtubules in WT and p62KO MEF were depolymerized and regrown as (A). These cells were then fixed and immunostained with anti-α-tubulin antibody. The amounts of polymerized tubulin were examined by fluorescence microscope.

CHAPTER V. FUTURE DIRECTIONS AND SUMMARY

Future Directions

In my dissertation research, I identified and mapped the interaction between the multi-functional protein SQSTM1/p62 and the cytoplasmic histone deacetylase HDAC6. I found that this interaction resulted in decreased HDAC6 activity. In p62KO MEFs, lack of p62/HDAC6 interaction led to increased HDAC6 activity and decreased acetylation of HDAC6 substrates. In p62 deficient background, I also studied the possible functional consequence in the aggresome-autophagy pathway and microtubule dynamics due to absence of this interaction. While these results suggested that p62/HDAC6 interaction may play important roles in regulation of protein aggregation and their autophagic degradation as well as microtubule dynamic properties, research questions towards fully understanding the mechanism and functional consequence of this interaction are addressed in the followings.

HDAC6 is a unique member of the histone deacetylase protein family with two deacetylase domains and predominant cytoplamsic localization (Verdel et al., 2000, Zhang et al., 2006). A number of cytoplasmic proteins have been shown to regulate deacetylase activity of HDAC6 through physical interaction

(Perez et al., 2009, Tokesi et al., 2010, Di Fulvio et al., 2011). In this study, I identified p62 as an additional HDAC6 interacting protein and I examined the role of p62 in modulation of HDAC6 activity. While I was able to demonstrate that p62 interacts with HDAC6 at its DD2 domain suggesting this physical interaction may be inhibitory to tubulin deacetylase activity, molecular details of this regulation are yet to be investigated. To characterize the inhibitory effect of DD2 binding to HDAC6 tubulin deacetylase activity, an in vitro tubulin deacetylase assay would need to be performed by incubation ofeither WT HDAC6 with p62 and purified tubulin or WT HDAC6 with purified tubulin. The prediction would be that addition of p62 prevents deacetylation of tubulin by HDAC6. To further characterize the binding site on DD2, mass spectrometry would need to conducted to identify residues responsible for this binding. To examine whether binding of these identified residues is inhibitory to HDAC6 tubulin deacetylase activity, an alanine scan would need to be performed by first generating the mutant with replacement of each identified residue with alanine through site-mutagenesis. The deacetylase activity of these mutants would need to be assessed by an *in vitro* tubulin deacetylase assay. Results from these experiments would deepen understanding of the mechanism by which p62 regulates HDAC6 tubulin deacetylase activity.

Both p62 and HDAC6 have been documented to play important roles in processing ubiquitinated protein aggregation and autophagic degradation (Kawaguchi et al., 2003, Bjorkoy et al., 2005, Pankiv et al., 2007, Lee et al., 2010a). Both proteins have a C-terminal ubiquitin-binding domain, thus can recruit ubiquitinated proteins to promote aggresome or inclusion body formation (Seigneurin-Berny et al., 2001, Ciani et al., 2003). Both p62 and HDAC6 also play crucial roles in recruitment of central autophagy

component, autophagosomes. P62 has a LIR that directly binds autophagosome protein LC3 (Pankiv et al., 2007). The complete relationship between HDAC6 and autophagosomes is yet to be identified. Nevertheless, both knockdown or pharmacological inhibition of HDAC6 have been shown to compromise recruitment of autophagosomes to aggresomes, indicating an essential role of HDAC6 in this process (Iwata et al., 2005b). Therefore, it is possible that p62 and HDAC6 may function redundantly in the aggresome-autophagy pathway. Interestingly, I showed that HDAC6 activity was elevated in p62KO MEFs, indicating p62 depletion may lead to augment of HDAC6 function. Moreover, expression of exogenous HDAC6 in p62KO MEFs recovered aggresome formation. These results support the idea that HDAC6 may compensate the functional defect in the aggresome-autophagy pathway in p62KO MEFs. More detailed studies need to be performed to examine if and how HDAC6 can replace p62 in regulation of critical steps in the aggresome-autophagy pathway.

While HDAC6 could play compensatory role to restore aggresome-autophagy function in p62-deficient cell model, it is also possible that HDAC6 and p62 may work in tandem to concentrate protein aggregates at aggresomes (Yao, 2010). Aggresome formation is dependent on motor-mediated transport of aggregates to MTOC (Kopito, 2000). In this model, p62 promotes ubiquitinated protein aggregation through its UBA and PB1 before transport to MTOC (Bjorkoy et al., 2005). These protein aggregates are then loaded onto dynein motors through the ZnF-UBP region of HDAC6 to form a cargo-HDAC6-dynein complex which is transported to MTOC (Kawaguchi et al., 2003). My identification of a clear p62-HDAC6 interaction indicated that proteins that bind p62 could be directly recruited to HDAC6-dynein complex through this interaction, thus

further supporting tandem participation of p62 and HDAC6 in aggresome formation. This seems to be especially important for aggresome formation of non-ubiquitinated substrates. In fact, a recent study revealed that HDAC6 activity is required for aggrsome formation of a non-ubiquitinated protein STAT5A_ΔE18 (Watanabe and Tanaka, 2011). Future studies should examine whether p62 serves as an adaptor between non-ubiquitinated proteins and HDAC6 for loading cargo to motor proteins. In addition, p62 has been shown to preferentially bind K63-ubiquitinated proteins (Seibenhener et al., 2004). It would also be interesting to postulate that p62-HDAC6's interaction may facilitate aggresome formation of K63-chain specific ubiquitinated proteins. Coincidently, K63-chain specific ubiquitination is considered to be a signal for aggresome formation as it has been identified in a number of ubiquitinated proteins accumulated at aggresomes (Olzmann et al., 2007, Tan et al., 2008, Lim and Lim, 2011).

Another interesting finding of my study was that tubulin acetylation decreased in p62KO MEFs. This is also the case in the neurons from p62KO mouse. Acetylation of tubulin has been shown to regulate a variety of physiological processes of neurons, such as neurite outgrowth, axonal transport, and mitochondrial transport (Reed et al., 2006, Chen et al., 2010, d'Ydewalle et al., 2011). Tubulin acetylation induced by HDAC inhibitors enhances binding and transport of motor proteins on microtubules, thus promoting neurite outgrowth and axonal and mitochondrial transport (Reed et al., 2006, Chen et al., 2010, d'Ydewalle et al., 2011). Dysfunction of these physiological processes in neurons is associated with neurodegenerative diseases. In particular, reduced axonal and mitochondrial transport has been observed in AD neurons (Stokin and Goldstein, 2006, Guo et al., 2013). It has been shown that mitochondrial transport defects can be

corrected when tubulin acetylation is induced by HDAC inhibitor (Kim et al., 2012). Interestingly, our laboratory's previous work has shown AD like phenotype in p62KO mice (Ramesh Babu et al., 2008). It is possible that decreased tubulin acetylation the p62KO model may negatively affect motor transport and consequently lead to malfunction of axons and mitochondria in neurons. These defects may contribute to pathogenesis of AD-like phenotype in p62KO mice. Future studies are needed to investigate this hypothesis.

Summary

Aggresome-autophagy is a critical degradation pathway responding to protein misfolding stress. The findings in this study support essential roles of p62 and HDAC6 in the aggresome-autophagy pathway. More importantly, these results provide insights regarding the possible models in which p62 and HDAC6 could work together to maintain the integrity of this pathway. In addition, for the first time, function of p62 is associated with microtubules. This connection is made by its interaction with HDAC6 and its modulation on HDAC6 activity. The results in this study implicate that presence of p62 may be critical for microtubule dynamics and motor trafficking. This would be a novel role of p62 which is important for microtubule-dependent cellular functions.

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